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General overview of results

The specific [^3H]flunitrazepam binding to neocortical and hippocampal membranes was measured following a 2 week treatment of rats with the benzodiazepine receptor antagonist flumazenil (FL; Ro 15-1788; 2.7 or 4 mg/kg/day in drinking water). The binding sites showed a gradually increasing density, which remained elevated for up to 24 and 72 hours after drug withdrawal. The dissociation constants (K_d) for the ligand remained largely unchanged. Most importantly, a strong uncoupling between the GABA and the benzodiazepine recognition sites was found in the cortex but not in the hippocampus, as measured by reduction in GABA enhanced [^3H]flunitrazepam binding to neuronal membranes from FL-treated animals killed 72 hours after drug withdrawal.

The behavioral correlates of chronic exposure of adult rats to FL (4 mg/kg/day x 21 days in drinking water) were as follows: on day 13 of drug treatment, there was a

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strong anxiolytic action, increased exploratory behavior (in the hole board test and the plus-maze test); these FL effects were coupled with inversely correlated ($P < 0.005$) defecation scores. In the Vogel's drinking-punishment test, on day 3, 6 and 10 after drug withdrawal, there was a significant ($p < 0.003$) "anticonflict" effect which was still present for at least 6 days after drug withdrawal. The FL exposure had no effect on the nociceptive threshold nor on the home-cage food and water consumption.

In the 12-arm radial maze, the FL-treated animals (4 mg/kg/day for 14 days in drinking water) showed an increase ($p < 0.002$) in non-appetitively motivated exploratory behavior (NAMEB), so labelled because it predominantly occurred in non-baited alleys facing the well illuminated "enriched environment" of the room center, as opposed to the baited alleys facing the "dull" room corner. The NAMEB emerged between day 5 and 7 of drug treatment, it continued to increase over the period of the remaining 8 days of drug treatment ($p < 0.002$), and peaked at day 3 after drug withdrawal ($p < 0.008$). The occurrence of NAMEB was inversely correlated with the urination/defecation scores ($p < 0.003$), and therefore, most likely, reflected the anxiolytic action of FL. Although in a stable and non-challenging environment, there were no differences between the control and the drug groups in the numbers of committed "working memory" errors, upon introduction of alley gates (to confine the animal for 10 sec to the center platform after an alley was explored), the working memory errors in the drug group remained low and unchanged ($p = 0.35$) relative to the preceding three trials, while the performance of the control group was disrupted, as indexed by an increase in the numbers of errors ($p < 0.004$). At day 7 of drug treatment, the emergence of NAMEB was associated with increased density and/or affinity of BDZ receptors in the cortex, hippocampus and brain stem, while three days after drug withdrawal, when the NAMEB reached its peak, there was a strong reduction in the GABA-enhanced [3 H]flunitrazepam binding to cortical synaptosomes.

In a water tank with one escape rope, tests conducted 24 hr after drug/vehicle withdrawal showed that the drug group, compared to controls, needed only 1/3 of the time to resolve the swim escape task. In the retention trial, 24 hr later, the control group barely matched the initial (24 hrs earlier) performance of the drug group. In a water T-maze equipped with two ropes (one anchored to the bottom and the other unanchored and more difficult to climb), on day 14 to 16 of daily FL/vehicle treatment, the FL group made highly significant progress, while the controls failed to show improvement in resolving the escape task ($p < 0.0001$).

Chronic FL (3-5 mg/kg in drinking water) protected the animals against amnesic effects of scopolamine. Chronic FL also strongly increased the total time the animals spent in Rapid Eye Movements (REM) sleep, without significantly changing the patterns of slow wave sleep; these effects were still present for 3 days after drug withdrawal. Chronic FL also facilitated the animals' habituation to novel and distracting environmental stimuli, a process known to depend on the cholinergic (muscarinic) transmission.

Taken together, the above actions of FL revealed a unique pharmacologic profile for a single drug: contrary to the benzodiazepine anxiolytics which impair vigilance, memory, cognitive and habituation processes, and contrary to the amphetamine-like stimulants that increase vigilance but prevent habituation processes and increase the central and peripheral effects of emotional stress,-- FL combines only the clinically favorable attributes of both classes of drugs, as it promotes the habituation processes to novel and distracting environmental stimuli, has anxiolytic actions coupled with increased vigilance, cognitive functions and learning.

On the basis of indirect but strong evidence, it has been postulated that the unique pharmacologic profile of FL is determined by drug-induced uncoupling between the GABA and the benzodiazepine recognition sites and resulting disinhibition of the normally present and tonic GABAergic control of the brainstem and forebrain cholinergic and aminergic systems. The enhanced aminergic functions may also be responsible for the known FL elevation of the brain oxygen utilization and anticonvulsant properties of FL. Hence, the unique pharmacologic profile of FL seem to represent a new class of nootropic drugs with a significant clinical potential.

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"ATTENTION SPAN, ANXIETY AND BENZODIAZEPINE RECEPTORS"*

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RESEARCH PAPERS published, in press or in preparation:

- 1) Marczyński, T. J. and M. Urbancic: Animal models of chronic anxiety and "fearlessness". Brain Res. Bull. 21(1988) 483-490.
- 2) Urbancic, M. and T.J. Marczyński: Chronic exposure to Ro 15-1788: differential effect on flunitrazepam binding to cortex and hippocampus. Eur. J. Pharmacol. 171(1989) 1-7.
- 3) Marczyński, T.J.: Adenosine presynaptic inhibition and transmitter "spillover": A new hypothesis of etiopathogenesis of narcolepsy. Medical Hypotheses 29(1989) 265-273.
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- 5) Marczyński, T.J., Urbancic, M. and M.A. Gadek: Chronic flumazenil (Ro 15-1788) enhances non-appetitive exploratory behavior of rats. Neuropharmacology, in press 1991, manuscript # A3117(B).
- 6) Urbancic, M., M. A. Gadek and T.J. Marczyński: Chronic flumazenil (Ro 15-1788) facilitates acquisition and retention of a swim-escape response in rats. Neuropsychobiology, 1991 in press, manuscript # 275.
- 7) Marczyński, T.J.: Neurochemical interpretation of cortical slow potentials as they relate to cognitive processes and a parsimonious model of mammalian brain: In: C. McCallum, Ed., Proceedings of the NATO Advanced Research Workshop on Slow Potential Changes in the Human Brain (held in Ciocco, Tuscany, Italy, May 13-16, 1990). Raven Press, New York, in press 1991.
- 8) Finnerty, M., T.J. Marczyński, H.J. Amirault, M. Urbancic and B.R. Andersen: Benzodiazepines inhibit neutrophil chemotaxis and superoxide production in a stimulus dependent manner; PK-11195 antagonizes these effects. Submitted to Immunopharmacology, January 2, 1991, manuscript # 861-91.
- 9) Urbancic, M., T.J. Marczyński and M.A. Gadek: Chronic flumazenil protects rats against amnesic action of scopolamine as tested by acquisition and retention of a swim-escape behavior. in preparation.
- 10) Szabo, I. and T.J. Marczyński: An unconventional low-noise preamplifier for multi-site recording of brain multiunit activity. In preparation
- 11) O'Connor, S., M. Urbancic, T.J. Marczyński, M. Radulovacki: Chronic Ro 15-1788 treatment increases REM sleep in rats. In preparation.

Abstracts

- 1) Urbancic, M. and T.J. Marczyński: Chronic exposure to Ro 15-1788 decreases GABA facilitation of ³H-Flunitrazepam binding in rat neocortical membranes. Soc. Neurosci. Abstracts 14(1988) 979.

2) Urbancic, M. and T.J. Marczyński: Anxiolytic effect resulting from chronic exposure to flumazenil (Ro 15-1788). Soc. Neurosci. Abstracts 15(1989) 669.

3) Urbancic, M. and T.J. Marczyński: Chronic flumazenil enhances learning of a swim-escape task in rats. Soc. Neurosci. Abstracts 16(1990) 1331.

DETAILED TECHNICAL PROGRESS REPORT

Abbreviations:

CNS - central nervous system; BDZ - benzodiazepine; Bmax - maximum number of binding sites; CBR - central, i.e. neuronal benzodiazepine receptor; DZ - diazepam; FL - Flumazenil (Ro 15-1788), a relatively neutral, neuronal BDZ receptor antagonist; FMLP - N-formyl methionyl-leucyl-phenylalanine; FNT - Flunitrazepam, a neuronal BDZ receptor agonist; GABA - gamma-amino butyric acid; K_D - dissociation constant; KLH - keyhole limpet hemocyanin; PBR - peripheral (non-neuronal) benzodiazepine receptor; PK-11195 - a peripheral, i.e. non-neuronal BDZ receptor antagonist; Ro 5-4864 - a peripheral, i.e. non-neuronal BDZ receptor agonist; SOD - superoxide dismutase;

PART I

Urbancic, M. and T.J. Marczyński: Chronic exposure to Ro 15-1788: differential effect on flunitrazepam binding to cortex and hippocampus. Eur. J. Pharmacol. 171(1989) 1-7.

Abstract

The time course of changes in specific [3 H]flunitrazepam binding following 2 weeks of treatment with the benzodiazepine antagonist Ro 15-1788 (2.7 and 4 mg/kg/day in drinking water) was studied in the rat neocortical and hippocampal synaptosomal membranes. Such a treatment produced regional increases in the density of benzodiazepine sites, which remained for up to 24 and 48 hours after drug withdrawal in the hippocampus and cortex, respectively; the dissociation constant (K_D) was unchanged. In addition, a significant reduction in GABA enhanced [3 H]flunitrazepam binding to cortical, but not to hippocampal, membranes from Ro 15-1788 - treated animals was found 72 hours after drug withdrawal. These data show that there is a regional difference in responses of the GABA/benzodiazepine receptor complex following chronic in vivo exposure to Ro 15-1788 and that, beside the increases in the maximal binding (B_{max}) of [3 H]flunitrazepam, the coupling between the GABA and the benzodiazepine recognition sites was also affected.

1. Introduction

The imidazodiazepine derivative, Ro 15-1788, a potent and selective benzodiazepine (BDZ) receptor antagonist (Hunkeler et al., 1981, Mohler and Richards, 1981) raises many questions regarding the mechanism of its action. Initially introduced as a neutral BDZ receptor antagonist, Ro 15-1788 appears to have anxiogenic or anxiolytic effects when administered at low doses (4-10 mg/kg) and high doses (20-50 mg/kg), respectively (Pellow and File, 1984).

Chronic treatment of adult rats with Ro 15-1788 (4 mg/kg/day in drinking water for 14 days) elevated the density of BDZ binding sites in rat neocortex and hippocampus, with no changes in the affinity of the receptor for BDZs (Medina et al., 1983). Perinatal exposure to Ro 15-1788 during ontogeny of BDZ receptors had a lasting anxiolytic effect in adult rats associated with an enduring increase in BDZ receptor number in the hippocampal formation (Marczynski et al. 1988; Marczynski and Urbancic, 1988). To investigate the influence of chronic Ro 15-1788 administration to adult rats on behavior (in progress), we had first to determine the time course of the Ro 15-1788 action on BDZ receptor regulation. We utilized [3 H]flunitrazepam ([3 H]FNTZ) binding and GABA potentiation of [3 H]FNTZ binding as probes of the GABA/BZD receptor complex. Our binding studies showed two effects of chronic administration of Ro 15-1788 on the GABA/BZD receptor complex: a) a short lasting increase in [3 H]FNTZ binding (24 and 48 h after drug withdrawal) to hippocampal and cortical membranes, respectively; and b) surprisingly, an attenuation of GABA enhancement of [3 H]FNTZ binding to cortical but not to hippocampal membranes.

2. Materials and methods

Adult male Sprague-Dawley rats (260-300 g), obtained from Bio-Lab Co (Saint Paul, MN), received Ro 15-1788 in the drinking water for 14 days. The average dose was 2.7 and 4 mg/kg/day. The control group received water with ethylene glycol (final concentration 0.5%), which was used to dissolve the drug. The rats were sacrificed by decapitation 4, 24, 48 and 72 hours after drug withdrawal and the cerebral cortex and the hippocampal formation were dissected and stored at -70°C . The tissues were homogenized in 10 volumes of ice cold 10% sucrose (w/w), using a glass homogenizer fitted with a teflon pestle, and centrifuged at $1\,000 \times g$ for 10 minutes. The resulting supernatant was centrifuged at $30\,000 \times g$ for 30 minutes. Using Polytron homogenizer, the pellet was resuspended in 20 volumes of 25 mM Tris-HCl buffer (pH=7.3 at 0°C) and centrifuged at $30\,000 \times g$ for 20 minutes. This procedure was repeated twice. The final membrane preparations were frozen and stored at -70°C for at least 20 hours. Prior to use, the membranes were thawed and suspended in assay buffer (25mM Tris-HCl) to give a protein concentration of 0.3-0.5 mg/ml. Protein concentrations were determined by the method of Lowry et al. (1951).

The measurement of total receptor binding was made by incubating 30 μl of homogenate in 25 mM Tris-HCl buffer at 0 to 4°C for 60 minutes in the presence of seven concentrations of [3 H]flunitrazepam (methyl- ^3H ; 81.8 Ci/mmol; Du Pont Company) ranging from 0.2 to 22 nM, and in a total volume of 0.1 ml. Nonspecific binding was measured in parallel incubations in the presence of excess (3 μM) unlabeled clonazepam and represented 5-10% of the total; it was subtracted from total binding to yield specific binding. In parallel incubations, 10 μM GABA and 200 mM NaCl were added to the assay mixture to enhance [3 H]flunitrazepam binding. The incubations were terminated by filtration under vacuum through Whatman GF/B filters. The radioactivity remaining on the filters was estimated by liquid scintillation counting.

In the dose-response experiments, membranes were washed five times prior to freezing, and various concentrations of GABA were added to the incubation medium immediately after the addition of [3 H]flunitrazepam (0.6 nM in final concentration).

The apparent K_d and the total number of binding sites (B_{max}) were estimated by Scatchard analysis, using a computer program LIGAND (Munson and Rodbard, 1980). The variability for each calculated slope was very small as reflected by the correlation coefficient ranging from 0.983-0.999. The significance of differences between control and treated groups was calculated using the Student's t-test. In order to evaluate the

GABA effect on [^3H]flunitrazepam binding to cortical and hippocampal membranes from Ro 15-1788 and vehicle exposed animals. analysis of variance (ANOVA) with repeated measures was used.

3. Results

3.1. Effect of chronic Ro 15-1788 treatment on benzodiazepine binding

In agreement with previous findings (Medina et al., 1983), the long-term administration of the benzodiazepine antagonist Ro 15-1788 (2.7 mg/kg/day in drinking water for 14 days) to adult rats significantly increased [^3H]FNTZ binding to neocortical and hippocampal membranes (table 1). Similar results are obtained in the experiments where the animals were treated with 4 mg/kg/day of Ro 15-1788 for 14 days (data not shown). The highest increase of Bmax for cortical receptors was observed 4 h after Ro 15-1788 withdrawal (+25%, $P < 0.02$); this upregulation gradually diminished and was not significant 72 h after drug withdrawal.

The upregulation of BDZ receptors in the hippocampus had a slightly different time course, as it reached the peak 24 h after Ro 15-1788 withdrawal (+13%, $P < 0.03$). There were no differences in the Kd values between the control and the Ro 15-1788 treated group.

TABLE 1a

Time course of BDZ receptor upregulation after chronic administration of Ro 15-1788 (2.7 mg/kg/day for 14 days). Using 0.2-22 nM [^3H]flunitrazepam, binding assays were carried out in synaptosomal membranes of the rat cerebral cortex and the hippocampal formation. For each drug group $n=5$, and for each control group $n=4$. Values represent means \pm S.E. * $P < 0.03$ compared to control (Student's t-test).

Time after Ro 15-1788 withdrawal (hours)	CORTEX		HIPPOCAMPUS	
	control	Ro 15-1788	control	Ro 15-1788
4 - Bmax (fmol/mg)	1226 \pm 81	1525 \pm 43*	1193 \pm 29	1222 \pm 18
Kd (nM)	1.3 \pm 0.03	1.3 \pm 0.04	1.3 \pm 0.06	1.3 \pm 0.01
24 - Bmax	1250 \pm 29	1394 \pm 49*	1193 \pm 17	1344 \pm 48*
Kd	1.3 \pm 0.04	1.2 \pm 0.03	1.1 \pm 0.03	1.1 \pm 0.01
48 - Bmax	1265 \pm 36	1402 \pm 34*	1198 \pm 34	1258 \pm 16
Kd	1.3 \pm 0.01	1.2 \pm 0.03	1.1 \pm 0.03	1.2 \pm 0.01
72 - Bmax	1238 \pm 58	1416 \pm 68	1111 \pm 89	1286 \pm 40
Kd	1.4 \pm 0.03	1.3 \pm 0.03	1.2 \pm 0.04	1.3 \pm 0.02

TABLE 1b

Time course of BDZ receptor upregulation after chronic administration of flumazenil, i.e. Ro 15-1788 (2.7 mg/kg/day for 14 days). Using 0.2-22 nM [3 H]flunitrazepam, binding assays were carried out in synaptosomal membranes of the rat cerebral cortex and the hippocampal formation. The increase in the maximal binding capacity (B_{max}) of [3 H]flunitrazepam binding is expressed as percent of control. For each drug group n=5, and for each control group n=4. *P<0.03 compared to control (Student's t-test).

Time after Ro 15-1788 withdrawal (hours)	[3 H]FNTZ receptor binding (% of control)	
	CORTEX (B _{max})	HIPPOCAMPUS (B _{max})
4	125*	103
24	112*	113*
48	111*	105
72	114	111

3.2. Effect of chronic Ro 15-1788 treatment on GABA facilitation of benzodiazepine binding

GABA (10 μ M) + NaCl (200 mM) enhancement of [3 H]FNTZ binding was assayed in the membranes from animals sacrificed 72 h after Ro 15-1788 withdrawal, e.g. at the time when no significant changes in the K_d and B_{max} values were observed. GABA+NaCl significantly decreased K_d values and marginally, but significantly increased B_{max} values in cortical and hippocampal membranes from both the vehicle or the Ro 15-1788 treated rats (table 2). As shown by the relatively small but significant (P<0.005) reduction of the dissociation constants, K_d, the increase of receptor affinity for [3 H]FNTZ in response to GABA+NaCl was comparable in both the vehicle and the Ro 15-1788 treated animals. Also, these increases were comparable in both the cortical and the hippocampal membranes. On the other hand, the GABA enhancement was found to be significantly reduced in the cortical membranes from Ro 15-1788 exposed animals: in the nontreated animals, GABA+NaCl significantly increased the B_{max} by 11% (P<0.01), while in the Ro 15-1788 treated rats this increase dropped to 4% (Fig.1 and table 2). In the hippocampal membranes, GABA+NaCl produced comparable increases of B_{max} values in both the vehicle and the Ro 15-1788 treated animals (by 8% and 5%, respectively). Although it is accepted that GABA increases affinity of BDZ receptors without changing B_{max} values (Martin and Candy, 1978; Karobath and Sperk, 1979; Regan et al., 1980), there are reports that concentrations of GABA greater than 10⁻³ M, in the presence or absence of NaCl, increase the B_{max} of [3 H]diazepam (Chiu and Rosenberg, 1979; Chiu and Rosenberg, 1982) and [3 H]flunitrazepam binding (Squires, 1981). Thus, the small but significant increase in B_{max} values might represent cooperativity of GABA and NaCl in facilitating [3 H]flunitrazepam binding.

TABLE 2

GABA (10 μ M) + NaCl (200 mM) enhancement of [3 H]flunitrazepam binding to synaptosomal membranes of vehicle-treated or Ro 15-1788-treated rats (4 mg/kg/day for 14 days in drinking water), sacrificed 72 hours after drug withdrawal.

EXPERIMENT	Bmax (fmol/mg prot) mean \pm S.E.	Kd (nM) mean \pm S.E.
CORTEX (n=5)		
(1) Control	1506 \pm 87	1.23 \pm 0.03
(2) Control+GABA+NaCl	1672 \pm 97 ^a	1.00 \pm 0.03 ^b
(3) Ro 15-1788	1656 \pm 58	1.15 \pm 0.03
(4) Ro 15-1788+GABA+NaCl	1718 \pm 72 ^c	1.00 \pm 0.01 ^d
HIPPOCAMPUS (n=5)		
(1) Control	1194 \pm 32	1.21 \pm 0.06
(2) Control+GABA+NaCl	1286 \pm 47 ^a	0.95 \pm 0.03 ^b
(3) Ro 15-1788	1288 \pm 26	1.08 \pm 0.03
(4) Ro 15-1788+GABA+NaCl	1358 \pm 40 ^c	0.92 \pm 0.01 ^d

^aP<0.01 vs. control (paired Student's t-test)

^bP<0.005 vs. control

^cP<0.04 vs. Ro 15-1788

^dP<0.005 vs. Ro 15-1788

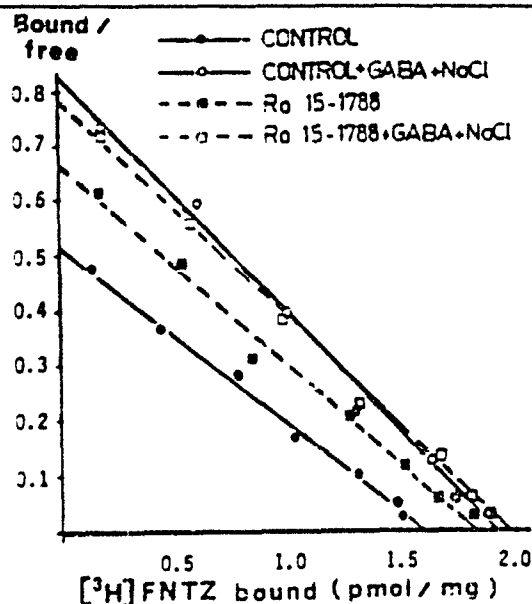


Fig. 1. Scatchard analysis of the effect of GABA (10 μ M) plus NaCl (200 mM) on [3 H]flunitrazepam binding to neocortical membranes from animals treated with vehicle (control) or Ro 15-1788 (4 mg/kg/day) for 14 days and sacrificed 72 hours after drug withdrawal. Notice the reduced GABA enhancement in membranes from Ro 15-1788 treated rats, as compared to the control. This illustration is based on a representative experiment.

Concentration-response curve of GABA enhancement of [3 H]flunitrazepam binding revealed a decrease in GABA effect on [3 H]FNTZ binding in the neocortex, but not in the hippocampus of Ro 15-1788 treated animals 3 days after drug withdrawal (Fig.2). In order to gain more insight into the nature of chronic effect of Ro 15-1788, the experiments were done in the absence of NaCl, which per se is known to potentiate benzodiazepine binding (Martin and Candy, 1978; Chiu and Rosenberg, 1980) and could influence the GABA effect. In the neocortical membranes from Ro 15-1788-treated rats, GABA facilitation of [3 H]FNTZ binding was strongly reduced, as compared to control [$F(1,12)=24.6$; $P<0.0005$, ANOVA test], while in the hippocampal membranes from the same animals GABA facilitation was not significantly altered [$F(1,12)=2.58$; $P=0.13$]. The same assays were carried out with cortical membranes prepared from animals sacrificed 24 h after drug withdrawal. A significant difference in the increase of [3 H]FNTZ binding in drug-treated group, as compared to control, was observed only for high concentrations of GABA (10^{-5} M to 3×10^{-4} M) (data not shown). The concentrations of GABA used in these experiments were without effect on non-specific [3 H]flunitrazepam binding.

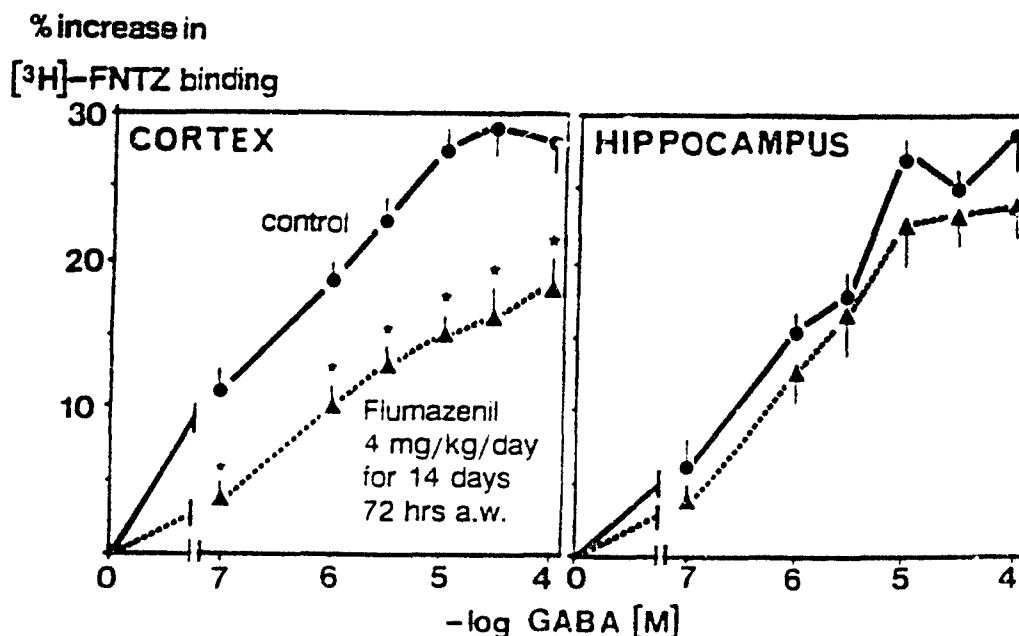


Fig. 2. Effect of GABA on [3 H]FNTZ binding to cortical and hippocampal membranes from control (o) and rats chronically exposed to Ro 15-1788 (Δ) (4 mg/kg/day for 14 days), 72 hours after drug withdrawal. The binding assays were performed without or with GABA ($0.1-100 \mu\text{M}$) and in the presence of 0.6 nM [3 H]flunitrazepam. Results are expressed as percent increase above basal values, i.e. in the absence of GABA. In the cortical membranes from Ro 15-1788 treated rats, GABA facilitation of [3 H]FNTZ binding was significantly reduced [$F(1,12)=24.6$; $P<0.0005$, ANOVA test], while in the hippocampal membranes there was no significant alteration in the GABA effect [$F(1,12)=2.58$; $P=0.13$]. Values represent means \pm S.E. of 7 animals/group.

4. Discussion

This study has investigated the time course of BDZ receptor regulation and the coupling between BDZ and GABA recognition sites in the neocortex and hippocampus of adult rat following chronic Ro 15-1788 treatment. Our results confirm the earlier observations (Medina et al., 1983) that a long-term administration of the BDZ antagonist Ro 15-1788 induced the upregulation in the number of BDZ receptors in the rat cerebral cortex and hippocampus, as measured by [3 H]FNTZ binding. In addition, our study demonstrates that the upregulation of BDZ receptors is short lasting, since it disappears 2 and 3 days after drug withdrawal in the hippocampus and cortex, respectively. Similarly, a recovery from BDZ receptor downregulation which followed chronic treatment with the BDZ agonist flurazepam, was also rapid and occurred within 24 hours (Rosenberg and Chiu, 1981). GABA potentiation of benzodiazepine binding provides information regarding the coupling between the BDZ and GABA receptors which is critical for understanding the pharmacologic and physiologic properties of the GABA/BDZ/(chloride ionophore) receptor complex. We have observed a significant reduction of GABA enhancement of [3 H]FNTZ binding to cortical membranes from animals chronically exposed to Ro 15-1788, and this effect seems to be independent of the BDZ receptor upregulation. The reduced GABA effect might have resulted from allosteric changes and/or modified functional interaction between GABA and BDZ recognition sites. Decreased GABA enhancement of [3 H]flunitrazepam binding was also found in cortical membranes from chronic diazepam-treated rats, without changes in BDZ receptor number or affinity (Gallager et al., 1984). Our data suggest that chronic receptor occupation not only with a BDZ agonist, but also with a BDZ receptor antagonist, may alter receptor configuration (or that of the entire GABA_A/BDZ receptor complex) that could account for a change in the coupling between GABA and BDZ recognition sites. Our data may also support the idea of Gonsalves and Gallager (1988) that Ro 15-1788 alters the coupling of elements of the GABA/BDZ macromolecular complex. This assumption, according to these authors, would account for their observations that GABAergic subsensitivity of the raphe neurons, developed during chronic diazepam treatment, may be reversed by a single systemic administration of Ro 15-1788, despite the presence of diazepam in the brain.

Considering the Ro 15-1788 effects, one can exclude several potential mechanisms. Since Ro 15-1788 has a short half-life in the rat brain (Lister et al., 1984), decreased GABA enhancement of [3 H]flunitrazepam binding in cortical membranes from drug-treated animals 72 h after drug withdrawal is most likely not related to the presence of Ro 15-1788 in our membrane preparations. Changes in endogenous GABA levels are unlikely to be responsible either, since the membranes were thoroughly washed. Considering the possibility that BDZ₂ sites are less sensitive than BDZ₁ sites to the effect of GABA (Corda et al., 1988), we can speculate that the upregulation of BDZ receptors, following chronic Ro 15-1788 treatment, is likely to affect predominantly the BDZ₂ receptors. Finally, it is still an open question whether in our study the endogenous ligand(s) and modulator(s) could have been cofactors in reducing the GABA enhanced benzodiazepine binding.

Our observation that GABA mediated enhancement of [3 H]flunitrazepam binding to hippocampal membranes, as compared to cortex, was not significantly affected by chronic Ro 15-1788 treatment, and the different degree and rate of BDZ receptor upregulation in the hippocampus and cortex, suggest a regional difference in sensitivity of the GABA/BDZ receptor complex to chronic Ro 15-1788 exposure. The regional heterogeneity observed following chronic exposure to Ro 15-1788 may reflect differences in the mode of coupling of the constituents of the GABA/BDZ receptor complex. Also, in different brain regions the same BDZ receptor subtype may be

differentially coupled to GABA receptors or the same receptor may be in a different modulating environment (Sieghart, 1988). In support of this view, quantitative autoradiographic measurement of BDZ receptor downregulation in various brain areas showed anatomical heterogeneity in the nervous system's response to chronic benzodiazepine exposure (Tietz et al., 1986).

In summary, two conclusions may be drawn from our results. First, it appears that the coupling between GABA and benzodiazepine receptors in the neocortex is affected not only by chronic exposure to BDZ agonist (Gallager et al., 1984, Mele et al., 1984), but also by chronic exposure to a BDZ antagonist, Ro 15-1788, as measured by the decreased ability of GABA to facilitate [³H]flunitrazepam binding in membranes prepared from the drug-treated rats. This suggests that a chronic displacement of an endogenously active substance(s) at the BDZ receptor, or its accumulation, may alter the interactions within GABA/BDZ receptor complex. Second, our results indicate that the cerebral cortex and the hippocampus are differently affected by chronic exposure to Ro 15-1788.

Three major observations warrant further investigations on the mode of Ro 15-1788 action: 1) changes in BDZ receptors described in the present study appear to be correlated with enhanced exploratory behavior of the animals (unpublished); 2) Ro 15-1788 may be useful in preventing the development of tolerance and dependence to chronically administered benzodiazepines (Gallager et al., 1986); and 3) surprisingly, Ro 15-1788 pretreatment has been found to prevent amnesia in mice induced by the cholinergic receptor antagonist, scopolamine (Lal et al. 1988).

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PART II

Urbancic, M., Gadek, M.A. and Marczyński T.J.: Chronic exposure to flumazenil: anxiolytic effect and increased exploratory behavior, *Pharmacol. Biochem. & Behavior*, 35(1990) 503-509.

Abstract

The aim of this study was to define the behavioral correlates of chronic exposure of adult rats to flumazenil (4 mg/kg/day x 21 days in drinking water). In the holeboard test, performed on day 13 of drug treatment, the animals showed a significantly greater interest for the holes under which objects were placed than for the holes without objects ($p < 0.03$), while there was no such difference in the control group. In the plus-maze test, the flumazenil-treated animals spent significantly more time on open arms and left less fecal boluses than the controls, when tested in the third week of treatment and 24 hours after flumazenil withdrawal. In the drinking-punishment test, conducted on day 3, 6 and 10 after drug withdrawal, the drug-exposed animals, following shock experience, did not significantly alter their unpunished drinking in subsequent trials, while the control rats significantly reduced ($p < 0.003$) their unpunished drinking. Also, the punished drinking revealed a significant "anticonflict" effect of prior exposure to flumazenil ($p < 0.006$) which was still observed 6 days after drug withdrawal. There were no group differences in the home-cage food and water consumption during flumazenil treatment; also, the drug treatment had no effect on nociceptive threshold. In summary, chronic treatment with a benzodiazepine receptor antagonist, flumazenil, increased exploratory activity and had a lasting anxiolytic effect.

Introduction

In 1981, it was discovered that an imidazodiazepine, Ro 15-1788, specifically antagonized the effect of benzodiazepines (BDZs) in a variety of biochemical, electrophysiological and behavioral tests (19, 28). Although originally considered as a "neutral" BDZ antagonist (2), evidence is accumulating that Ro 15-1788 (flumazenil) is not devoid of intrinsic pharmacological actions. Flumazenil has been shown to have behavioral actions of its own which can be classified as either agonist or inverse agonist, depending upon the test condition and the dose used. In rats, acute administration of flumazenil has anxiogenic effects in the social interaction test in doses of 10 mg/kg, i.p., but not in doses of 4 and 20 mg/kg, i.p. (10, 12), in the punished drinking test, if injected in doses of 20 and 30 mg/kg, i.p. (13), and in a test of food (8 mg/kg, i.p.) and water consumption (10 mg/kg, i.p.) in novel environment (13, 17).

Some of the animal tests of anxiety have failed to detect any action of flumazenil; for instance, the elevated "plus maze" [10-20 mg/kg and 4 mg/kg, i.p. (1, 32)], and the punished drinking test, when injected in doses of 2 mg/kg IV (6, 27) or in doses of 25 mg/kg, i.p. (36), and in doses of up to 100 mg/kg, p.o. (3). On the other hand, flumazenil (4-20 mg/kg, i.p.) was found to increase exploratory behavior in the holeboard test, without affecting locomotor activity (11), and to exhibit a weak antiaversive action (35 mg/kg, i.p.) as measured by the latency of escape reaction to electrical stimulation of the periaqueductal grey matter (22). The latter actions of flumazenil could be classified as BDZ agonist-like. In human subjects, flumazenil, also showed a BDZ-like agonist actions in several psychophysiological tests, such as EEG

spectra, blood pressure, tremor, reaction time task, etc (18). Generally in rodents, relatively low single doses of flumazenil (4-10 mg/kg) appear to have anxiogenic effects, while higher doses (20-50 mg/kg), seem to have weak diazepam-like, anxiolytic effects (7,30).

Little is known about the effects of chronic administration of flumazenil (FL). In rats, perinatal exposure to flumazenil, 3 mg/kg for 3 weeks administered in drinking water to pregnant and subsequently lactating dams during ontogeny of BDZ receptors, had a lasting effect in adult, 5 months old offspring, as reflected by increased BDZ receptor numbers in the hippocampal formation and the associated more efficient and "fearless" goal-directed behavior, compared to offspring perinatally exposed to diazepam or the drug vehicle (23,24). In contrast, a higher dose of flumazenil (20 mg/kg SC), administered to pregnant rats during the last week of gestation, resulted in a decrease of neocortical BDZ binding and a lower seizure threshold, as measured in 18 days old offspring (14).

Chronic exposure of adult rats to flumazenil (4 mg/kg for 14 days in drinking water) increased the number of BDZ and beta-carboline binding sites (26). Using the same 2 week treatment regimen, we have confirmed the above observations, and, in addition, we have found a significant decrease in GABA facilitation of flunitrazepam binding to neocortical membranes of flumazenil exposed rats, a change that was still present 72 hours after drug withdrawal (35).

Prompted by the above receptor studies, the aim of our present investigation was to evaluate the behavioral correlates of chronic 21 day exposure of adult rats to flumazenil, by focusing on the time period between day 13 through day 21 of daily drug administration, and including the time period of 10 days after drug withdrawal. Toward this end, the following behavioral tests were used: 1) those that are believed to measure anxiety, such as the elevated "plus-maze" test (31) and the drinking-punishment test (37); 2) the holeboard test that measures both the exploratory and the locomotor activity (9); 3) tests that measure the threshold to painful stimuli: the tail-flick test and the tail-shock vocalization test; and finally 4) we evaluated the food and water intake and the changes in body weight of the chronically treated animals.

Materials and Methods

Animals and drug administration

Male Sprague-Dawley rats (Bio-Lab Corp. Saint Paul, MN), weighing 270-290 g at the beginning of the study, were used (for control group n=12, for flumazenil-treated group n=11); they were singly housed in an air-conditioned room with a 14 hr light/10 hr dark cycle (lights on at 0600hr), and were allowed free access to food (Purina-Chow) and water. After the average daily water consumption during a 7 day period had been ascertained, flumazenil (Ro 15-1788, 4 mg/kg/day) was dissolved in ethylene glycol (5 ml per 1000 ml of tap water) and administered in drinking water for 3 weeks. The drug was generously provided by Dr. Peter Sorter from the Hoffmann LaRoche Co. (Nutley, N.J.). The control group received a comparable volume of the drug vehicle in drinking water. The rats tended to drink less per kg of body weight parallel with an increase in their body weight, e.g. the mean volume of the consumed water for both groups on day 1 equaled 131.3 ml/kg, while on the last day 21 of treatment this value equaled 97.0 ml/kg. The volume of consumed water was measured every 24 hours and any significant change in water consumption was compensated by adjusting the drug concentration to optimize the intended dose of 4 mg/kg/day. The average daily dose of flumazenil equaled 4.0 ± 0.2 SD mg/kg.

Apparatus and Procedures

All experiments were carried out between 0800 and 1200 AM. After each trial, fecal boluses were counted and removed from the test arena and the floor was thoroughly cleaned. The behavior in the plus-maze was recorded on video tape and later analyzed by two investigators, one of whom had no knowledge of the drug state of the animals.

1) Holeboard test. The apparatus was a wooden box 60 x 60 x 36 cm, with four equally spaced holes in the floor, each 3.5 cm in diameter; various objects were placed under two of the holes (9). The level of illumination was 30 scotopic lux. The infrared photocells, placed under each hole, monitored the number of the animal's head-dips and the time spent head-dipping, while the photocells placed in the walls measured the locomotor activity. Each rat was tested for 5 min on day 13 of chronic drug treatment.

2) Elevated plus-maze test. The apparatus (31) consisted of four horizontal wooden arms: two opposite open arms (50 x 11 cm), and two opposite enclosed arms (50 x 11 x 40 cm), connected by a central platform (11 x 11 cm). The maze was elevated 50 cm above the floor. In a variant of this test, the access to two enclosed arms was blocked allowing the animal either to enter the open arms or to stay in the partially enclosed center platform; the elevation of the maze was reduced from 50 cm to 25 cm above the floor. In both variants of the test, the level of illumination was 22 and 30 scotopic lux for protected and open arms, respectively.

The first test was conducted on day 13 of drug or vehicle treatment, immediately after the animal was subjected to a 5 minute test in the holeboard box, and therefore had time to habituate to handling and novel environment. The remaining tests in the plus-maze (from day 15 through 21 of drug treatment and 24 hours after drug withdrawal) were carried out without prior holeboard test, i.e. each animal was transferred directly from its home cage to the plus-maze. After the first six daily 7.5 min trials in a regular plus-maze, the animals received additional three daily 5 min tests in the plus-maze with the enclosed arms blocked. The repeated daily tests enabled us to assess the effect of increasing familiarity with a test arena on the animal's behavior. Also, closing the entrance to protected arms, followed by water deprivation, provided insight into a potential role of novel exogenous and endogenous stimuli on the animal's behavior. The number of entries and total time spent in each type of arms were scored. There is a convincing evidence that validates this simple test as a sensitive tool for measuring the exploratory behavior and the anxiolytic or anxiogenic action of drugs (31).

3) The drinking-punishment test was performed in a clear plexiglas cage, the same in which the rats were housed, but without bedding and equipped with a stainless steel floor. The metal drinking spout and the floor were connected to a constant current shock generator and to the drinkometer (Columbus International, Inc, Columbus, Ohio). Two days prior to the initiation of drug treatment and 24 hours after water deprivation, rats were tested for the latency to start drinking and number of unpunished licks per one minute. On the basis of these tests, the animals were divided into two comparable groups for flumazenil and vehicle treatment. On day 3, 6 and 10 after drug withdrawal, the 44 hr water-deprived animals were tested for 1 min of unpunished drinking, followed by a 5 min period of punished drinking. After every 20 licks, 0.35 mA current was delivered to the drinking tube. Measures were: the latency to the first lick, the number of licks in unpunished and punished periods, and the number of shocks received.

4) Tail-flick reflex latency. The rat's tail was placed under an intense light source and the latency for tail withdrawal was measured. The time between the onset of the light stimulus and the triggering of the photodetector was defined as the tail-flick

latency. The test was carried out on day 6 after drug withdrawal.

5) Tail-shock vocalization test. Two aluminum foil bands were placed around the midportion of the rat's tail and alternating current, ranging from 25 to 60 mA, was applied to the tail for 0.1 sec (for details, see ref. 4). The lowest current intensity at which the rat vocalized was used as the threshold for painful stimuli. The test was carried out on day 8 after drug withdrawal.

Statistics

The differences in behavioral scores within a group over daily trials and between two groups of animals over time during and after chronic administration of the drug or drug vehicle were ascertained using the BMDP software for one- or two-way analysis of variance (ANOVA) with drug treatment, or drug/vehicle withdrawal and days as factors, or ANOVA with repeated measures. If the distribution of the data met the requirements, the t-test was used to compare two samples of data. If the data did not fulfill the assumptions of ANOVA, they were logtransformed. In instances of significant departures from normal distribution of the data or dependence of variances on the means, the nonparametric tests were used to ascertain the within-group differences over time (the Wilcoxon Matched Pairs Rank Sum test). The differences between two groups in the numbers fecal boluses left in the test arena were analyzed by chi square test.

Results

1) Holeboard test

On day 13 of chronic treatment with flumazenil, there were no significant differences in the mean number of head-dips between the groups (control, 15.7 ± 1.8 SE; drug, 17.5 ± 3.1 SE) nor in the time spent head-dipping (control, 25.2 sec ± 3.3 SE; drug, 23.6 sec ± 2.8 SE). However, the flumazenil-treated animals showed a significantly greater interest for the holes where the objects were present than for the holes without objects [$F(1,20)=5.5$, $p<0.03$], whereas in the control group the time spent head-dipping at the holes with or without objects was not significantly different [$F(1,22)=2.8$, $p=0.11$].

Flumazenil had no effect on locomotor activity (control: 526 ± 40 SE; drug: 547 ± 36 SE).

2) Plus-maze test

The animals were tested daily, beginning on day 13 through day 21 of continuous flumazenil treatment (except day 14), and 24 hrs after drug withdrawal and water deprivation (W1; abscissa; Fig. 1 top). Trial 1 was performed immediately after each animal spent 5 min in the holeboard box, while the subsequent trials were done without prior exposure to the holeboard, in order to see whether or not the change in the procedure (with or without acclimation in the holeboard) would have a differential effect on behavior of the control and the drug-treated group in the plus-maze test. The results showed that indeed this procedure disclosed significant differences between the flumazenil-treated group and the control group. In trial 1 that immediately followed the holeboard exposure (day 13 of drug treatment), there were no significant differences between the control and the drug-treated group in the mean percent time the animals spent on the open arms (ordinate of Fig. 1 top). However, in trial 2 conducted 48 hrs later (day 15 of drug treatment) the control group showed a decrease in the mean percent time spent on open arms, as compared to trial 1 ($p<0.02$, paired t-test) and this exploratory behavior continued to be significantly depressed (s) through trial 4 (day 17 of drug treatment; $p<0.02$).

Conversely, the behavior of the drug-treated rats was not significantly suppressed by the lack of prior exposure to the holeboard box, and on day 17 and 18 (trial 4 and 5, respectively), the percent time spent on open arms was significantly higher in the drug-treated rats, as compared to the control ($p < 0.05$ and $p < 0.03$, respectively; two-tailed t-test).

A comparison of both groups over trials 2 through 6 showed a significantly increased exploratory behavior of flumazenil-treated rats (two-way ANOVA; $F(1,105) = 7.68$; $p = 0.007$). On day 19 (trial 6), the control rats showed signs of habituation to the test apparatus and their time spent on open arms was not different from that of drug-treated animals and was also comparable to their post-holeboard (trial 1) performance. Judging from the time course of exploratory behavior of either group, in the control group, the aversion to the open arms remained unchanged over time (trials 2 through 6; $F(4,44) = 1.8$; $p = 0.15$), while in the flumazenil-treated group, there was a significant increase in exploratory behavior over time [$F(4,40) = 5.31$; $p = 0.0016$].

In the next trial 7 (day 20 of drug treatment), the animals were confronted with a novel situation in the plus-maze design, since both entrances to the enclosed arms were blocked. This change disclosed group differences, since it triggered a stronger exploratory response in the drug treated animals, relative to controls ($p < 0.04$; two-tailed t-test). On the last day 21 of drug treatment (trial 8), the difference between the two groups was not significant, but the next day (trial 9), i.e. 24 hrs after drug withdrawal and water deprivation, the drug-exposed rats again spent significantly more time on the open arms than the controls ($p < 0.04$).

The mean number of fecal boluses the animals left after each test (ordinate of Fig. 1 bottom panel) reflected the group differences in the time course of habituation to the testing procedure: in the initial 3 trials, the 12 control rats in $3 \times 12 = 36$ individual trials left a total of 28 boluses, and the ratio of 28/36 was comparable to that for 11 drug exposed rats: 31/33 ($X^2 = 0.13$; $df = 1$; $p = 0.72$). However, in subsequent 5 daily trials, the sum total ratio of 73/60 in the control group was higher than the ratio of 14/55 for the drug group ($X^2 = 20.79$; $df = 1$; $p < 10^{-5}$).

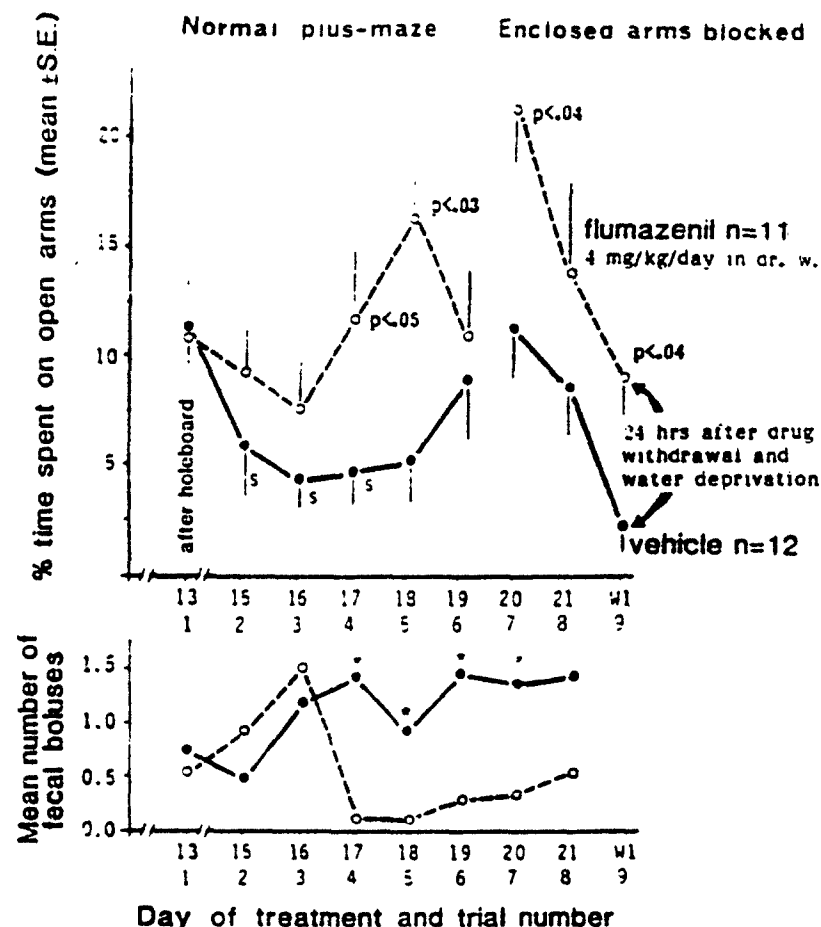


Fig. 1. **Top:** Enhanced exploratory behavior of rats during chronic 21 day treatment with flumazenil (4 mg/kg/day in drinking water), as measured by the group mean percent time spent on open arms of the elevated plus-maze (ordinate). Rats were tested on day 13 and day 15 through 21 of drug or vehicle treatment, and 24 hours after drug or vehicle withdrawal (W1; abscissa). Trial 1 (day 13) was conducted immediately following a 5 min. holeboard test which, to some extent, habituated the animals to handling and novel environment, and, as a result, both groups showed comparable exploratory behavior.

However, in subsequent trials without prior holeboard exposure, the control group was significantly (s ; $p < 0.02$; paired t -tests) more reluctant to explore the open arms than on the post-holeboard trial, while the drug-treated group was not significantly inhibited, and on day 17 (trial 4) and day 18 (trial 5), the differences between the groups were significant ($p < 0.05$ and $p < 0.03$; two-tailed t -tests). After the entrance to the enclosed arms had been blocked (trial 7 through 9), the drug-treated group spent more time on open arms than the control group ($p < 0.04$). In the last trial, conducted 24 hr after drug withdrawal and water deprivation, again a significant difference between the two groups emerged, the drug-exposed group showing more exploratory behavior than the control group ($p < 0.04$).

Bottom: The mean numbers of fecal boluses (ordinate) the animals left in the plus-maze after each of 1 through 8 trials (abscissa). Note that the drug-treated group, in trials 4 through 8 almost ceased defecating and the difference in defecation score between the two groups was highly significant ($p < 10^{-5}$).

When comparing the time course of the daily defecation scores with the time course of the mean percent time the animals spent on open arms over the 8 sequential trials (Fig. 1, the bottom and the top panels, respectively), it became apparent that there was an inverse relationship between these two measures. Moreover, by plotting for each trial the mean numbers of fecal boluses left by the animals in the plus-maze versus the mean percent time the animals spent on open arms (Fig. 2), a significant negative linear regression was obtained ($r=-0.64$; $n=16$; $p=0.007$).

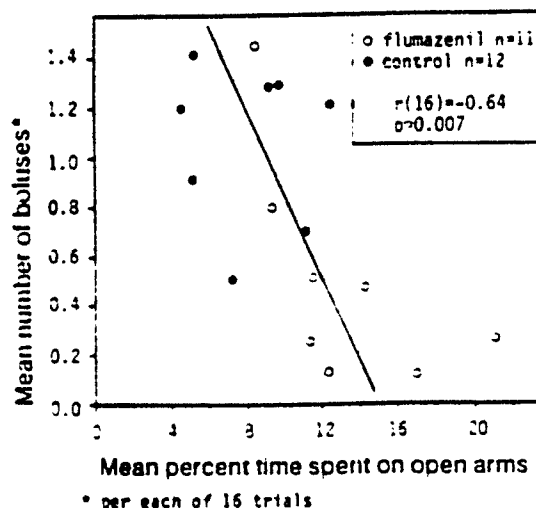


Fig. 2. Significant inverse relationship ($r=-0.64$; $p=0.007$) between the mean number of fecal boluses left by the control group and the drug-treated group after each of 8 trials in the plus-maze (ordinate) and the mean percent time each group spent on open arms of the plus-maze (abscissa).

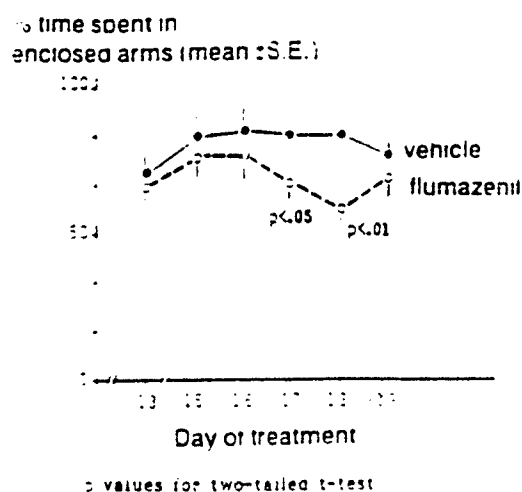


Fig. 3. Effect of chronic treatment with flumazenil on the mean percent time spent in the enclosed arms of the elevated plus-maze.

In addition, the drug-treated animals, compared to the control group, spent significantly less time in the enclosed arms, when tested on day 17 and 18 of continuing treatment (trial 4 and 5; Fig. 3).

The increased exploratory activity of the flumazenil-treated animals was not only expressed by the increased percent time spent on open arms, but also by significantly higher number of total arm entries [$F(1,126)=9.43$; $p<0.003$, for trials 1 through 6]. Also, in trial 7, when the entrances to the enclosed arms were blocked, and in trial 9, following a 24 hr water deprivation and drug withdrawal, the drug-treated group made significantly more entries into the open arms than controls (Table 1).

Table 1
Mean (\pm S.E.M.) number of entries and time spent on two open arms (5 min. test)

time of testing	control	Ro 15-1788 (4mg/kg/day)
day 20		
number	3.8 \pm 0.8	6.0 \pm 0.5 *
time (sec)	36.2 \pm 8.5	63.3 \pm 7.7 *
day 21		
number	2.5 \pm 0.7	3.4 \pm 0.8
time (sec)	26.3 \pm 8.8	42.2 \pm 14.5
24 hr water deprivation		
number	0.7 \pm 0.3	2.6 \pm 0.8 **
time (sec)	8.3 \pm 3.4	28.0 \pm 8.1 *

* $p<0.05$, ** $p<0.03$ for difference from control (Student's t-test)

3) Drinking-punishment test

Chronic 21 day exposure to flumazenil had a significant "anticonflict" effect on both unpunished and punished drinking (ordinate in Fig. 4 left and right, respectively), as tested on day 3, 6 and 10 following drug withdrawal (abscissa). The response magnitudes in the unpunished pre-drug trial and in trial on day 3 after drug/vehicle withdrawal, reflected a shock-naive state of the animals, and the group mean responses were virtually identical. However, the first shock the animals experienced on day 3 of vehicle withdrawal, significantly reduced the unpunished drinking in the control group during the subsequent test ($p<0.003$; Wilcoxon Matched Pairs Signed Rank test); the second shock experience on day 6 of vehicle withdrawal further deepened this suppression on day 10. On the other hand, the unpunished drinking in the drug group, compared to the naive condition, was not significantly altered after shock experience (ns; $p=0.57$ and $p=0.17$ for day 6 and 10, respectively), and, on day 10 of drug withdrawal, there was a significant difference in unpunished drinking between the control and the drug exposed-group ($p<0.006$; two-tailed t-test). The ANOVA also showed that in control group, the shock experience significantly reduced unpunished drinking [$F(2,30)=14.6$; $p<0.00004$], while in the drug-treated group the unpunished drinking was not altered by shock experience [$F(2,27)=0.98$; $p=0.39$].

Punished drinking (right panel of Fig. 4) that followed 1 min. of unpunished drinking also revealed "anticonflict" effect of prior exposure to flumazenil, as tested in 5 min trials on day 3 and 6 after drug or vehicle withdrawal. The drug-exposed

animals. on the average, received 10.9 ± 1.8 SE shocks and 19.3 ± 4.2 SE shocks on day 3 and 6, respectively, while the control animals received only 4.5 ± 0.8 SE and 6.8 ± 1.3 SE shocks, respectively, and these differences were significant ($p < 0.04$ and $p < 0.006$, respectively; two-tailed t-test).

The latency to the first lick was not affected by drug withdrawal, nor by the shock experience.

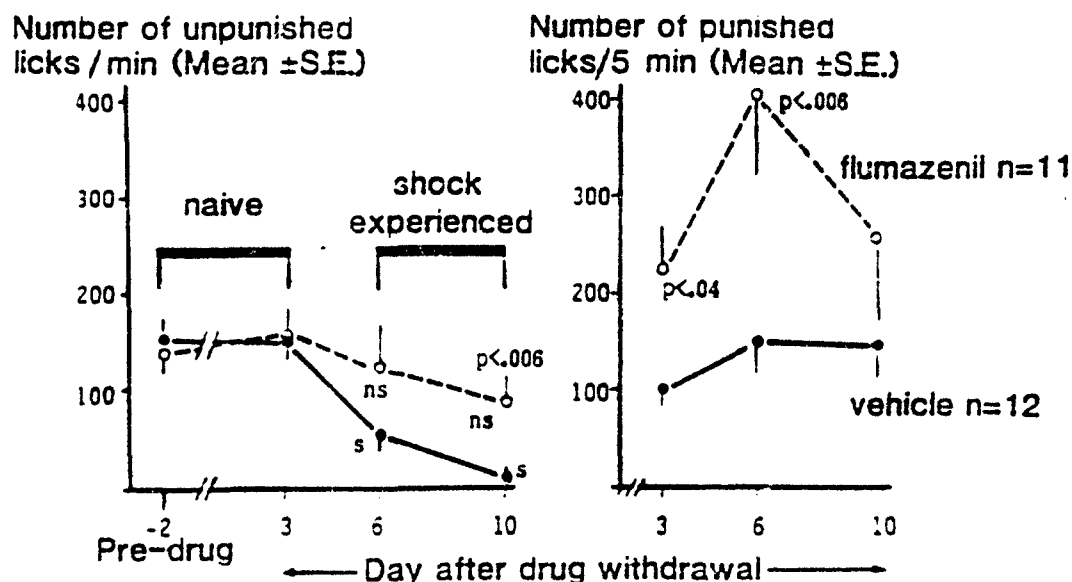


Fig. 4. The "anticonflict" effect of chronic exposure to flumazenil on the mean number of unpunished and punished licks (ordinate; left and right panel, respectively) plotted for days 3, 6 and 10 following drug withdrawal (abscissa).

A 1 min. of unpunished drinking was followed by 5 min. of punished drinking (0.35 mA shock after each 20 licks), except for the pre-drug test (2 days prior to initiation of the treatment). Each test was carried out after 44 hr water deprivation. In four trials of unpunished drinking (left panel), the condition of the animals can be divided into a shock-naive (pre-drug and day 3 after drug or vehicle withdrawal), and a shock-experienced condition (day 6 and 10 after drug or vehicle withdrawal).

Note that, in the control group, shock experience significantly (s) inhibited unpunished drinking, compared to each of the two naive state trials ($p < 0.003$; Wilcoxon test), while shock experience had no significant effect (ns; $p = 0.14$) on the drug exposed group; on day 10 of drug withdrawal, there was a highly significant difference between two groups ($p < 0.006$; two tailed t-test). Also, the punished drinking (right panel) was significantly enhanced on day 3 and 6 after drug withdrawal ($p < 0.04$ and $p < 0.006$, respectively; t-test), as compared to the control group.

4) Tail-flick test and tail-shock vocalization test

The latency of the tail-flick reflex and nociceptive threshold, measured by the tail-shock vocalization test, were not altered in the rats treated with flumazenil, as compared to controls, on day 6 and 8 after drug withdrawal (Fig. 5).

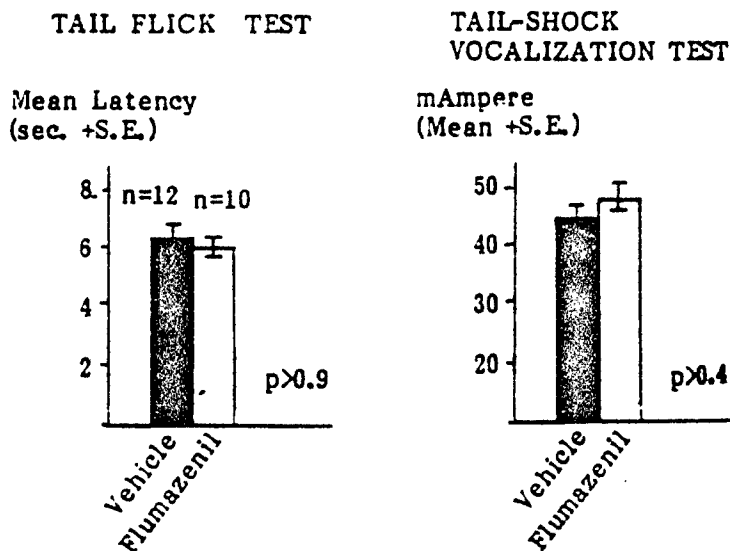


Fig. 5. Lack of antinociceptive effect of the chronic flumazenil treatment, measured on day 6 (tail-flick test) and day 8 (tail-shock vocalization test) after drug withdrawal, i. e. during the time when a significant "anticonflict" effect of the prior drug exposure was observed.

5) Food and water consumption

On the average, the water intake in the drug-treated group (112.8 ± 18.0 SD ml/kg/24 hrs) over the time period of 21 days was not significantly different from the vehicle-treated group (119.8 ± 13.7 SD ml/kg/24 hrs), [$F(1,16)=1.64$; $p=0.22$; ANOVA with repeated measures]. Also, over the same time period, the daily food consumption was not significantly different in both groups [$F(1,16)=0.32$; $p=0.58$]; it equaled 15.4 ± 2.6 SD g/200 g body weight in the control group, and 15.0 ± 1.6 SD g/200 g body weight in the drug group.

Discussion

We have observed a moderate anxiolytic effect of chronic exposure to BDZ antagonist, flumazenil, and, paradoxically, this effect was followed by an even more pronounced anxiolytic effect observed for several days after drug withdrawal. The following results indicate anxiolytic effects of chronic flumazenil treatment:

First, toward the end of the third week of flumazenil treatment and 24 hrs after drug withdrawal, the animals spent more time on open arms of the elevated plus-maze than the control group; such behavior was observed by other investigators following acute or chronic administration of BDZ agonist, such as chlordiazepoxide and diazepam (31), but not after acute treatment with flumazenil (32).

Second, in the plus-maze test, the defecation/urination scores, believed to reflect a temporary imbalance in the autonomic nervous system caused by emotional responses to novel environmental stimuli (34), were significantly decreased in

flumazenil-exposed animals, as compared to controls. The fact that the exploratory behavior of the animals in the plus-maze was inversely correlated with the defecation scores, indicate that the drug-exposed animals, compared to controls, were less emotional and their autonomic nervous system was more stable when challenged by novel environmental stimuli. Actually, in most instances, it was a challenge, environmental or endogenous, that unmasked the behavioral group differences: i) the initial use of the holeboard box for acclimation and then abandoning this procedure; ii) blocking the entrances to the enclosed arms of the maze; and iii) water deprivation, each triggered significantly stronger exploratory reaction in the drug group.

The unaltered locomotor activity and the total time spent head-dipping in the holeboard test found by us during chronic flumazenil treatment, have also been described in rats chronically treated with BDZ agonist, chlordiazepoxide and diazepam (31). Thus, one may be tempted to ascribe the above mentioned anxiolytic effects of chronic flumazenil to its weak BDZ agonist action. There is, however, a fundamental difference between the typical BDZ agonists and flumazenil: while the BDZ agonists, upon termination of chronic treatment, cause withdrawal symptoms that are indicative of emotional tension and anxiety (1, 20, 29), flumazenil withdrawal, in the present study, was characterized by unabated, if not potentiated, anxiolytic effect lasting at least 10 days after drug withdrawal as measured in the drinking-punishment test. Hence, we can conclude that the anxiolytic or "anticonflict" action of chronic flumazenil cannot be explained by its presumable weak BDZ agonist properties.

Three issues should be considered when searching for a plausible explanation of our observations:

- 1) the synthesis and the levels of a potential endogenous ligand(s) for BDZ receptors,
- 2) conformational changes within GABA/BDZ receptor complex, and
- 3) the influence of chronic flumazenil on other neurotransmitter and/or modulator systems.

Endogenous ligand(s). A polypeptide, labeled as a diazepam binding inhibitor (DBI), endowed with an anxiogenic, "proconflict", or "inverse-agonist"-like action, has been isolated from rat brain homogenates (16). It was suggested that DBI and/or its biologically active fragments could be stored and protected from further degradation in specific cellular compartments (8). Also, polypeptides from the bovine and human brain have been isolated that display a significant sequence homology with DBI (25). It is thus conceivable that a chronic occupation of BDZ recognition sites by flumazenil prevents the physiologic action of DBI, if this peptide and/or its active fragments are released when the animal is challenged by "intimidating" and/or stressful stimuli. In such a condition, flumazenil can be expected to have a stabilizing effect on emotional responses normally triggered by DBI, without having any significant effects of its own. Such a scenario of flumazenil action is compatible with our results, since, as mentioned above, differences between the flumazenil-treated animals and the controls did indeed emerge in response to novel and challenging stimuli. However, the apparent lack of anxiogenic effect of flumazenil withdrawal in the plus-maze test and the drinking-punishment test does not support the theory of flumazenil "protection" of BDZ receptors from DBI or its active fragments.

On the other hand, an endogenous BDZ agonist has been recently purified from bovine brain, and benzodiazepine-like immunoreactivity was detected in human brain from subjects who have not been exposed to BDZs (33). The purified substance was identified as the N-desmethyldiazepam, known as a common metabolite of BDZs, whose elimination half-life ranges between 50-100 hrs and has a tendency to accumulate in the body after prolonged BDZ treatment (15). A chronic competition of flumazenil with the endogenous

BDZ-like receptor ligand during the treatment could be expected to cause effects opposite to those observed in the present study. However, accumulation of a BDZ-like endogenous ligand during flumazenil treatment could, in theory, overcome the receptor blockade by flumazenil and displace the antagonist from BDZ receptors, thus producing an anxiolytic effect. An increased synthesis and release of a BDZ-like ligand could account for the anticonflict effects following drug withdrawal.

Conformation changes in BDZ/GABA receptor complex. The mechanism of behavioral changes reported here, may be more complex than the consequences of a competition of flumazenil for BDZ receptors. It has been suggested that flumazenil, in vitro, is especially efficient in shifting the BDZ receptor to the high-affinity conformation and that such an activity would mask the effects of agents known to enhance binding of benzodiazepine agonists, such as GABA, NaCl or pentobarbital (5). We have found a significant decrease in GABA enhancement of [³H]flunitrazepam binding to rat neocortical BDZ receptors that was still present 3 days following a two week flumazenil treatment (35), and this finding may reflect a conformation change in the BDZ/GABA receptor complex and altered coupling between the GABA and the BDZ recognition sites caused by flumazenil.

The upregulation of the central BDZ receptor density found after chronic flumazenil treatment (26), does not persist after drug withdrawal: it may last for up to 24 and 48 hr in the hippocampus and neocortex, respectively, the affinity remaining unchanged (35). Thus, in the present study, the significant anxiolytic effect of flumazenil observed 10 days after flumazenil withdrawal, can not be explained by alteration in the number nor affinity of BDZ receptors.

Possible interaction with the cholinergic system. As already mentioned, the increased exploratory behavior of the flumazenil-treated animals in the plus-maze test emerged whenever a change in the testing procedure and/or environment was introduced. This tendency may be explained by faster habituation and increased vigilance in the drug-treated animals. Such an interpretation is compatible with the suggestion made by other investigators (21) that flumazenil enhances vigilance and memory by antagonizing the suppressant influence on the cholinergic system normally exerted by the BDZ-like endogenous ligand(s) acting via the GABA/BDZ receptor complex.

In conclusion, on the basis of our current knowledge of the GABA/BDZ receptor complex, none of the above discussed mechanisms, if considered alone, can fully explain the anxiolytic effects of chronic flumazenil treatment and the apparent absence of withdrawal symptoms.

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PART III

CHRONIC FLUMAZENIL ENHANCES NON-APPETITIVE EXPLORATORY BEHAVIOR OF RATS
Marczynski, T.J., Urbancic, M. and Gadek, M., *Neuropharmacology*, 1991 in press

Abstract.

The effects of chronic administration of the benzodiazepine (BDZ) receptor antagonist, flumazenil (FL; Ro 15-1788; 4 mg/kg/day for 14 days in drinking water) on performance of adult rats in the 12-arm radial maze were studied. Relative to controls, the FL-treated animals showed an increase ($p < 0.002$) in non-appetitively motivated exploratory behavior (NAMEB), so labelled because it occurred in 88% of instances in non-baited alleys facing the well illuminated "enriched environment" of the room center as opposed to the baited alleys facing the "dull" room corner. The NAMEB emerged between day 5 and 7 of drug treatment, it continued to increase over the period of drug treatment ($p < 0.002$), and reached its peak at day 3 after drug withdrawal ($p < 0.008$; longer duration was not investigated). The occurrence of NAMEB was inversely correlated with the urination/defecation scores ($p < 0.003$), and therefore, most likely, reflected the anxiolytic action of FL. During drug/vehicle treatment, the control and the drug group made comparable numbers of "working memory" errors ($p = 0.17$). However, upon drug withdrawal and introduction of alley gates (to confine the animal for 10 sec to the center platform after an alley was explored), the working memory errors of the drug-exposed rats remained unchanged ($p = 0.35$) relative to the preceding three trials, while the performance of the control group was disrupted, as indexed by an increase in the numbers of errors ($p < 0.004$). At day seven of drug treatment, the emergence of NAMEB was associated with increased density and/or affinity of BDZ receptors in cortex, hippocampus and brain stem, while three days after drug withdrawal, when the NAMEB reached its peak, there was a reduction in GABA-enhanced [^3H]flunitrazepam binding in cortex.

Introduction

The imidazodiazepine flumazenil (FL; Ro 15-1788) is a potent and selective displacer of the benzodiazepine (BDZ) binding in vivo and in vitro (Hunkeler, Mohler, Pieri, Polc, Bonetti, Cumin., Schaffner, and Haefely, 1981; Mohler and Richards, 1981). This relatively neutral BDZ antagonist (File and Pellow, 1986), when administered in drinking water to pregnant and then lactating dams, enhanced the ontogeny of brain BDZ receptors, resulting in a progeny with enriched BDZ receptors in the hippocampal formation. When compared to controls, the adult progeny was more efficient in goal-directed behavior in the Radial Arm Maze, particularly if challenged by novel and "intimidating" environmental stimuli (Marczynski and Urbancic, 1988; Marczynski, Hawkins, Swann, Krivograd, Patel and Dugich, 1988). If administered to adult rats, FL (3-4 mg/kg/day for 14 to 21 days in drinking water) also upregulates cortical and hippocampal BDZ receptors (Medina, Novas and deRobertis, 1983; Marczynski and Urbancic, 1988) but, contrary to the perinatally-induced enduring effect, this upregulation lasts only for several days after drug withdrawal and is associated with uncoupling between the BDZ and the GABA recognition sites in the neocortical, but not in the hippocampal, neuronal membranes, as indexed by a reduction in the GABA-potentiation of flunitrazepam binding ("GABA shift") when assayed 72 hrs after FL withdrawal (Urbancic and Marczynski, 1989; see also Miller, Greenblatt, Roy, Gaver, Lopez and Shader, 1989). These receptor changes were associated with increases in the animals' vigilance, curiosity (holeboard test) and exploratory behavior (elevated Plus-Maze test), while the anxiolytic action (social interaction test and the Vogel's punished drinking test) was still significant for at least 10 days after drug withdrawal (Urbancic, Gadek and Marczynski, 1990).

Paradoxically, FL is rapidly metabolized to an inactive carboxylic acid which is eliminated as a glucuronide (Hunkeler, 1988), and, if given in a relatively large dose (10 mg/kg i.p.), FL's half-life time in the rat brain is only 16 min and, 80 min after injection, FL is no longer detectable using the high performance liquid chromatography (Lister, Greenblatt, Abernethy and File, 1984). Thus, during chronic FL treatment, the 5-7 day latency of the receptor and behavioral effects (the latter outlasting for at least 10 days the drug treatment) are not likely to result from the presence of the drug per se, but from relatively enduring conformational changes in the GABA_A/BDZ/(chloride ionophore) receptor complex and/or changes in dynamics of the endogenous ligand binding and synthesis. Hence, the aim of the present study was to ascertain behavioral changes of rats in the Radial Arm Maze during chronic FL treatment (4 mg/kg/day for 14 days in drinking water) and during 3 days following drug withdrawal. The latter period is of particular interest for two reasons: i) this is the time when the behavioral effects of chronic FL remain unabated for several days after drug withdrawal; and ii) in the absence of FL, the altered GABA_A/BDZ/(chloride ionophore) receptor complex is presumably no longer occupied by FL and therefore "free" to interact with endogenous angiogenic (Guidotti, Forchetti, Corda, Konkell, Bennet and Costa, 1983; Ferrarese, Alho, Guidotti and Costa, 1987; Medina, Pena, Novas, Paladini and de Robertis, 1987) and anxiolytic "endonepine" ligands (Sangameswaran and De Blas, 1988). In the present study, unusually robust exploratory behavior emerged between day 5 and 7 of chronic FL treatment. Thus, a supplementary BDZ receptor binding assay was carried in another group of rats which were not used in maze experiments but were treated for 7 days with FL or the vehicle and killed 6 hr or 24 hrs after drug withdrawal.

Materials and Methods

Animals and general procedures. Male Sprague-Dawley rats (Bio-Lab Corp., Saint Paul, MN), 70 days old and weighing 280-320 g at the beginning of the study, were used (for control group n=11, for FL-treated group n=12); they were singly housed in an air-conditioned room with a 14-hr light/10-hr dark cycle (lights on at 0600 hr). In order to find the appropriate drug concentration in drinking water, the mean daily water and food consumption during a 10 day prior to experiments had been ascertained; these data served as criterion for subdividing the animals into two comparable groups. Prior to trial 1, the animals were food deprived for 24 hr, and subsequently received in their home cages only 80% of their normal food requirement (Purina Chow) which per day and 200 g body weight equaled 8.21 g + 0.29 SD in the control group, and 8.22 g + 0.18 SD in the drug group; there was no difference between the groups (p=0.97; ANOVA). The average daily water intake per 200 g body weight equaled 17.4 + 2.7 SD ml and 16.2 + 2.4 SD ml in the control and the drug group, respectively, and these differences were not significant (p=0.2; t-test and Mann-Whitney test, thus allowing more meaningful conclusions from changes in the urination/defecation scores presumably indexing the animals' emotional reactions (Broadhurst, 1969; Sepinwall and Cook, 1982).

Inadvertent alterations within the GABA_A/BDZ/(chloride ionophore) receptor complex may occur and have behavioral consequences, if the animals, prior to experiments, are not fully habituated to handling (Biggio, Concas, Serra, Salis, Corda, Nurchi, Crisponi and Gessa, 1984; Trullas, Havoundjian, Zamir, Paul and Skolnick, 1987) or experience emotional stress (Antelman, Knopf, Kocam, Edwards, Ritchie and Nameroff, 1988). Thus, the animals were habituated to handling during seven consecutive days prior to the maze trials.

For six days prior to the maze trial, the animals received "Fruit Loops" in

their home cages, a sugar-coated cereal (Kellogg Co., Battle Creek, MI) which was eagerly consumed and subsequently used as bait in the radial maze.

Drug administration. FL was kindly provided by Dr. Peter Sorter (Hoffmann-La Roche Co., Nutley, NJ) and was dissolved in ethylene glycol (5 ml per 1000 ml of tap water) and administered in drinking water for 2 weeks. The volume of consumed water was measured every 24 hours, and any significant consumption change was compensated by adjustment of the drug concentration. The average daily dose of FL over the period of 14 days equaled $3.9 \text{ mg/kg} \pm 0.23 \text{ SD}$. The control group received a comparable volume of the drug vehicle in drinking water.

Test apparatus. The wooden radial maze (elevated 5 cm above the floor) consisted of a central platform (60 cm in diameter) and 12 symmetrically arranged arms, each 62 cm long, 12.5 cm wide and 25 cm high. From trial 7, the top of each arm was covered with a metal screen, as some animals tended to escape from the maze. At the entrance to each arm, there was a guillotine gate controlled by the experimenter via strings and pulleys. Between each gate and the metal screen on top of each arm, there was a 4x4 cm gap, and this was the only place through which a rat in the center platform could climb over the closed 20 cm high gate to gain access to an alley or to the top of the screen that covered the maze. A sugar-coated cereal bait was placed at the end of each arm 1 through 8 and served to test the "working memory" (Olton and Pappas, 1979); baits were hidden behind small partitions, so the rats were not able to see the reward from the center of the maze. Arms 9 through 12 were never baited and served to test the "reference memory".

Experimental design. The maze trials were run under the standard fluorescent light conditions, between 0800 a.m. and 0400 p.m. After each trial the defecation/urination scores were determined and the maze floor was thoroughly cleaned. The time of day for each animal's test was rotated through the 20 daily trials. Trial 1 through 3 were conducted in drug free animals; each animal, facing the center platform, was placed in the unbaited arm number 10, and was allowed to remain in the maze until all baits were collected or 15 min elapsed, whichever came first. Trials 4 through 17 were run in the drug or vehicle-treated animals. Trials 18 through 20 were run in drug/vehicle withdrawn animals. In these three trials, the animals were temporarily confined to the center platform by closing for 10 sec all 12 guillotine gates immediately after the animal collected the bait or explored the alley and returned to the center platform.

Scoring of behavior. The animal was allowed to remain in the maze until all baits were collected or the allotted time elapsed, whichever came first. The animal's behavior was observed on a videomonitor, and all trials were videotaped for future replay and analysis by two investigators in a manner that precluded knowledge of the drug treatment any animal had received.

The following behaviors were scored: 1) the latency to exit the first alley to which the animal was placed at the onset of trial, after the gates were opened; 2) the latency to consume 6 baits; 3) the latency to consume all 8 baits; 4) the number of collected baits during first 8 choices; 5) the number of "working memory" errors before collecting all 8 baits, i.e. the number of re-visited alleys from which the animal had already removed the bait; 6) the number of the "reference memory" errors, i.e. the number of entries into the 4 alleys which were never baited (alleys 9 through 12); 7) the frequency of exploratory behaviors, such as: with paws over the edge of the 25 cm high wall, lifting the body and hind legs above the floor in order to gain view of objects on the floor outside the maze or escaping from the maze; climbing to the top of an alley wall and walking on the screen that covered the maze;

climbing above the closed 20 cm high guillotine gate to access a closed alley; nudging the bottom edge of the gate, lifting it and crawling under into a closed alley; and 8) the number of fecal boluses and the amount of urine the animal left in the maze were integrated into a defecation/urination index, as follows: for each fecal bolus or urine pool of more than 0.5 ml, the animal was scored 50 points; for each urine deposit of 0.3 to 0.5 ml (approximately 6 to 10 drops), or less than 0.3 ml, the animal was scored 10 and 5 points, respectively.

Benzodiazepine receptor assay

Membrane preparation. The rats were chronically treated for 7 and 14 days with flumazenil (FL; 4 mg/kg/day in drinking water); control rats received comparable volumes of the drug vehicle. The animals were sacrificed by decapitation and the cerebral cortex, the hippocampal formation, thalamus, and the brain stem were quickly dissected on ice and stored at -70°C . The synaptosomal membranes were prepared as previously described (Marczynski et al., 1988; Urbancic and Marczynski, 1989).

Ligand binding assay. The measurement of total receptor binding was made by incubating 30 μl of homogenate in 25 mM Tris-HCl buffer at 0 to 4°C for 60 minutes in the presence of seven concentrations of [^3H]flunitrazepam (methyl- ^3H ; 81.8 Ci/mmol; Du Pont Company) ranging from 0.2 to 22 nM, and in total volume of 0.1 ml. Nonspecific binding was measured in parallel incubations in the presence of excess (3 μM) unlabeled clonazepam and represented 5-10% of the total; it was subtracted from total binding to yield specific binding. In the assays where [^3H]flunitrazepam binding was enhanced by addition of GABA or GABA+NaCl, neocortical membranes (washed five times prior to freezing) were incubated with various concentrations of GABA or GABA + 200 mM NaCl, added into the incubation medium immediately after the addition of [^3H]flunitrazepam (0.6 nM). The incubations were terminated by filtration under vacuum through Whatman GF/B filters, using a multiprobe cell harvester (Brandel Biomedical Research, Inc., Gaithersburg, MD). The radioactivity remaining on the filters was estimated by liquid scintillation counting.

In the BDZ receptor binding studies, the apparent dissociation constant (K_d) and the total number of binding sites (B_{max}) were estimated by the Rosenthal-Scatchard analysis, using a computer program LIGAND (Munson and Rodbard, 1980). The variability for each slope was small, as the linear correlation coefficient ranged from 0.983 to 0.999, indicating the presence of one receptor type. The differences in the mean B_{max} and K_d were estimated using the t-test.

Statistics. Using the BMDP statistical package and the VAX computer, the differences in behavioral scores between two groups of animals were ascertained using a repeated-measures two-way analysis of variance (ANOVA) with drug/vehicle treatment, or drug/vehicle withdrawal, as one factor and consecutive days as the second factor. This approach was chosen since our preliminary studies showed that treatment duration was a significant factor in gradually altering the GABA_A/BDZ/(chloride ionophore) receptor complex and behavior. If the variances of the mean values did not fulfill the assumption of independence from the means, the data were subjected to the square root or logarithmic transformation. If the transformed data failed to meet the symmetry assumption of the distribution of variances, the degrees of freedom and the p values were adjusted according to the Greenhouse-Geisser method. In order to compare the animal's performance in three trials before and three trials after drug/vehicle withdrawal, one-way ANOVA with repeated measures and contrast, and/or the Wilcoxon Rank Sum test and the Mann-Whitney test were used.

Results

Latency to exit first arm. The maze trials were initiated by placing each rat in the unbaited arm #10, and during the pre-drug period (trials 1 through 3) there was no difference between two groups in latency to exit the arm; however, there was a significant effect of days on the same measure, i.e. the rats tended to reduce the time spent in the first arm during the first three trials [$F(2,42)=4.95$, $p<0.01$]. This tendency was no longer significant during drug administration [trials 4 through 17; $F(12,252)=1.60$, $p=0.09$]. There was no difference between the groups during drug treatment [$F(1,21)=1.35$, $p=0.26$], nor after drug withdrawal [$F(1,121)=3.38$, $p=0.08$].

Latency to collect 6 baits. No significant differences between the groups were found prior to, during, and after drug/vehicle withdrawal. In both groups, this measure significantly decreased during the first three pre-drug daily trials [$F(2,42)=50.49$, $p<0.0001$], and continued to do so during the drug period, [trials 4 through 17, $F(12,252)=10.77$, $p<0.0001$]. There was no group difference following the introduction of gates/drug withdrawal period [trials 18-20; $F(1,21)=2.04$, $p=0.17$].

Latency to collect all 8 baits. During the three pre-drug trials, the latency to consume all 8 baits significantly decreased in both groups [$F(2,42)=14.74$, $p<0.0001$], and this trend continued during the drug/vehicle period [days 4 through 17, $F(12,252)=16.55$, $p<0.0001$]. The drug group, relative to control, did not significantly differ in trials 4 through 17 [$F(1,21)=1.64$, $p<0.21$]. The introduction of gates plus drug/vehicle withdrawal (trials 18 through 20) significantly increased the latency to consume all 8 baits in both groups [control group, $F(1,10)=104.13$, $p<0.0001$; drug group, $F(1,11)=30.10$, $p=0.0002$; ANOVA with contrast], when compared with the preceding trials 15 through 17, but there were no significant differences between the two groups [$F(1,21)=0.53$; $p=0.47$].

Number of collected baits during first 8 choices. This measure significantly increased over time in both groups for the 3 day pre-drug period [$F(2,42)=25.96$, $p<0.0001$], and during drug/vehicle treatment [day 4 through 17; $F(12,252)=8.03$, $p<0.0001$]. There was no effect of drug treatment [$F(1,21)=0.60$, $p=0.45$] nor drug withdrawal/introduction of gates [$F(1,21)=2.53$, $p=0.13$] on this measure.

Errors of "working memory" in the maximum allotted time or until all 8 baits were collected whichever came first (Fig. 1 top). These errors, i.e. the number of re-visited alleys from which the rat had already collected a bait, was not affected by drug treatment, relative to control. In both groups, the number of errors significantly decreased over the drug/vehicle treatment period [trials 4 through 17; $F(12,252)=10.43$, $p<0.0001$], and there was no group difference [$F(1,21)=2.01$, $p=0.17$]. During the most challenging three day period of drug/vehicle withdrawal and the introduction of gates, compared to the preceding three trials, the drug group made no significantly more errors [$F(1,11)=0.94$, $p=0.35$, contrast ANOVA repeated measures; $p=0.45$ in the Wilcoxon Sign Rank test], while the performance of control rats strongly deteriorated in response to this challenge and their errors markedly increased [$F(1,10)=13.84$, $p<0.004$; contrast ANOVA repeated measures; $p<0.008$, Wilcoxon Sign Rank test].

Errors of "reference memory" i.e. visits to unbaited alleys.

a) in first 8 choices. In both groups, the numbers of errors in the first 8 choices

significantly decreased during the 14 day period of drug/vehicle administration [$F(12,252)=5.63$, $p<0.0001$], and there was no difference between the groups [$F(1,21)=0.19$, $p=0.67$]. Also, the drug withdrawal plus introduction of gates, did not reveal any significant differences between the groups [$F(1,21)=1.22$, $p=0.28$].

b) in maximum allotted time or until all 8 baits were consumed whichever came first. The number of errors, were not significantly higher in the drug-treated group during the period of drug administration [trials 4 through 17, $F(1,21)=3.08$, $p=0.09$]. The number of errors decreased in both groups [$F(12,252)=9.01$, $p<0.001$], but there was no day x treatment interaction [$F(12,252)=1.63$, $p=0.08$]. In the most challenging trials 18 through 20, there was no difference between the two groups [$F(1,21)=0.05$, $p=0.83$], although relative to the preceding 3 trials 15 through 17, the numbers of errors were significantly increased in both groups [drug group, $F(1,11)=15.58$, $p=0.002$; control group, $F(1,10)=37.03$, $p=0.0001$].

Exploratory behavior (Fig. 1 middle). In 88% of instances, the exploratory behaviors (the rearing animal, with front paws over the wall edge, lifting the body to gain sight of the floor and objects outside the maze, climbing over the wall and/or to the screen that covered the maze, also climbing over the alley gait or nudging it with head, lifting and crawling under the gate. etc.), occurred in the four unbaited alleys that faced the middle part of the well illuminated room equipped with furniture, while the baited alleys, facing the "dull" walls of the room corner, apparently were not attracting the animals' attention, despite that this area should be preferable for escape and hiding.

During the initial 3 day pre-drug period, this behavior marginally increased in both groups [$F(2, 42)=3.32$, $p=0.05$], but there was no difference between the two groups [$F(1, 21)=0.94$, $p=0.34$]; the Fisher's exact test showed comparable results ($p=0.3$). However, during the following 14 day period of FL administration, the number of exploratory episodes strongly increased in the drug-treated group, relative to the control group [$F(1,21)=12.92$, $p<0.002$]; by the Fisher's test, $p<0.00001$). The drug withdrawal/introduction of gates did not change the conspicuous group differences [$F(1, 21)=8.57$, $p<0.008$], the exploratory behavior of the drug group peaking in the last trial, while the control group showed virtually no motivation for exploring the extramaze environment.

Defecation/urination scores (Fig. 1 bottom). These scores were not different between the groups during the three days prior to drug administration [$F(1,21)=0.76$, $p=0.39$]. Also, if one considers the whole period of drug/vehicle treatment (days 4 through 17), the scores in the drug group did not significantly differ from those of the controls [$F(1,21)=0.27$, $p=0.61$]. During three days after drug withdrawal/introduction of gates, the increased scores in the drug group approached significance [$F(1,21)=3.88$, $p=0.06$]. However, if one considered the last 8 trials (days 12 through 20), the mean daily score for the drug group was more than 10 times lower than that for the control group (0.8 ± 0.52 S.E.M. vs. 8.1 ± 2.3 S.E.M.; $p<0.007$, Mann-Whitney test).

An approximate 7 day period of drug treatment was necessary for the behavioral changes to emerge, and the high scores continued unabated during the time period of drug withdrawal/introduction of gates, while the scores in the drug group remained virtually scoreless and unaffected by these novel and challenging conditions.

Pairing the group mean daily urination/defecation scores with the corresponding mean numbers of exploratory episodes over trials 4 through 20 revealed a significant inverse correlation between these two measures ($R=-0.50$; $p<0.003$; Spearman coefficient of rank correlation).

Efficiency index (Ei). In order to compare in a more general manner the animals' performance during the last three most challenging trials, for each animal the working memory errors and 1/10 of the defecation/urination score were added, and the numbers of exploratory episodes were subtracted from this sum. The reciprocal value of this sum yielded the Ei whose mean values for the drug group equaled 0.38 ± 0.10 SE and was 2.4 times greater than the mean Ei for the control group (0.16 ± 0.02 SE; $p < 0.002$, Mann-Whitney test).

Receptor changes

Reduction in GABA potentiation of [3 H]flunitrazepam binding. In animals killed 72 hrs after FL withdrawal, the GABA enhancement of [3 H]flunitrazepam binding to neocortical membranes was significantly reduced (Table 2; Fig. 2), thus confirming our earlier results (Urbancic and Marczyński, 1989).

Post-hoc [3 H]flunitrazepam binding assay. A separate group of rats, not used in maze experiments but of comparable age, received FL (4 mg/kg for seven days), while controls received the vehicle. In animals killed 4 hrs after drug/vehicle withdrawal, there was a significant increase in the numbers of BDZ receptors in the hippocampal formation. In animals killed 24 hrs after drug/vehicle withdrawal, there was a significant receptor affinity increase in the neocortex and the brainstem membranes (Table 2 below).

Table 1

Effect of 7 day exposure to flumazenil (4 mg/kg/day in drinking water) on the total number of benzodiazepine receptors (Bmax) and dissociation constant (Kd) in the hippocampus, neocortex, thalamus and brain stem of rats sacrificed 4 and 24 hours after drug withdrawal

	Time after flumazenil withdrawal			
	4 hours		24 hours	
	Bmax(fmol/mg)	Kd(nM)	Bmax(fmol/mg)	Kd(nM)
HIPPOCAMPUS				
control	1098 \pm 60	1.25 \pm 0.11	1128 \pm 80	1.22 \pm 0.07
Flumazenil	1214 \pm 50*	1.26 \pm 0.08	1156 \pm 80	1.27 \pm 0.11
CORTEX				
control	1447 \pm 124	1.23 \pm 0.04	1598 \pm 109	1.26 \pm 0.11
Flumazenil	1580 \pm 128	1.25 \pm 0.08	1654 \pm 150	1.12 \pm 0.03*
THALAMUS				
control	696 \pm 31	0.98 \pm 0.08	723 \pm 50	0.96 \pm 0.06
Flumazenil	728 \pm 37	1.01 \pm 0.04	709 \pm 80	0.99 \pm 0.07
BRAIN STEM				
control	1260 \pm 123	1.44 \pm 0.20	1280 \pm 78	1.40 \pm 0.03
Flumazenil	1288 \pm 103	1.31 \pm 0.07	1328 \pm 115	1.27 \pm 0.05**

* different from control ($p < 0.03$, two-tailed t-test); ** different from control ($p < 0.002$); for all groups $n=5$; values are means \pm SD

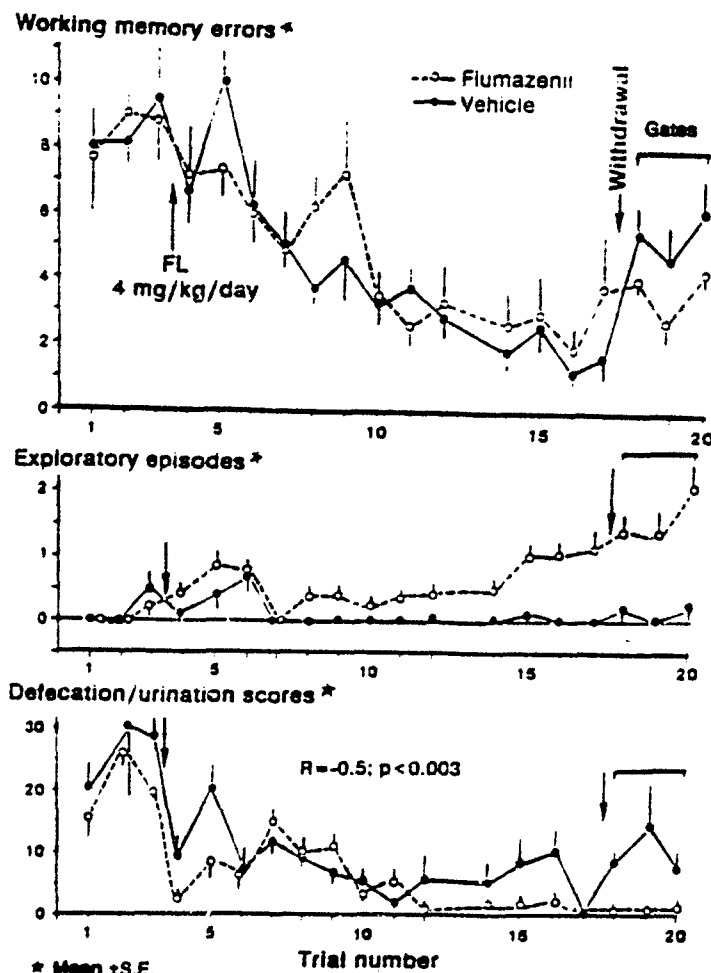


Fig. 1. Rat performance in the Radial Arm Maze during chronic 14 day administration of flumazenil and during subsequent three days of drug withdrawal (abscissa; trials 4 through 17 and 18 through 22)

Top: numbers of "working memory" errors (ordinate); in both groups, the number of errors decreased over trials 4 through 17 ($p < 0.0001$) and there were no group differences ($p = 0.17$). The introduction of gates and drug withdrawal revealed a significant difference between two groups: the performance of the control animals, relative to the preceding three days 15 through 17, was strongly impaired ($p < 0.004$), while the flumazenil-exposed animals were not significantly affected by this change in experimental procedure ($p = 0.35$).

Middle: numbers of exploratory episodes (ordinate). During drug administration (trials 4 through 17), relative to controls, the frequency of exploratory episodes increased ($p < 0.002$). Following the introduction of gates and drug withdrawal, this trend continued ($p < 0.0001$).

Bottom: Mean daily defecation/urination scores (ordinate); during drug administration (trial 4 through 17), if one considers the whole period of drug/vehicle treatment, there were no differences between the drug and the control group ($p = 0.61$). However, if one considers the last 8 trials (trial 12 through 20), the mean daily score for the drug group (0.8 ± 0.52 S.E.M.) was more than 10 times lower than that for the control group (8.1 ± 2.30 S.E.M.; $p < 0.007$, Mann-Whitney). Pairing the daily group mean defecation/urination scores with the corresponding daily mean numbers of exploratory episodes for the control and the drug exposed groups over trials 4 through 20, revealed a significant inverse correlation between the two measures (Spearman coefficient of rank correlation, R).

Table 2

Effect of GABA and chloride ions on clonazepam displaceable basal [^3H]flunitrazepam binding to neocortical membranes obtained from rats treated with flumazenil (4 mg/kg) and drug vehicle in drinking water for 14 days and killed 72 hours after drug withdrawal

Synaptosomal membranes were incubated at 0°C for 1 hr in the presence or absence of the NaCl (200 mM) with 0.6 nM [^3H]flunitrazepam and different concentrations of GABA in 25 mM Tris-HCl buffer (pH=7.3 at 0°C). Values are means (\pm SEM) of four to seven separate experiments, each performed in triplicates.

Additions		[^3H]FNTZ specific binding (% of basal binding)	
GABA	NaCl	CONTROL	FLUMAZENIL
10 μM	-	128 \pm 2.5	115 \pm 1.1 ^a
100 μM	-	128 \pm 2.9	116 \pm 2.1 ^b
100 μM	200 mM	136 \pm 1.9	138 \pm 4.2
300 μM	-	135 \pm 5.0	122 \pm 1.4 ^c
300 μM	200 mM	143 \pm 5.1	134 \pm 2.0

^a $p < 0.002$, ^b $p < 0.03$, ^c $p < 0.05$ versus the % increase in respective control by Student t-test

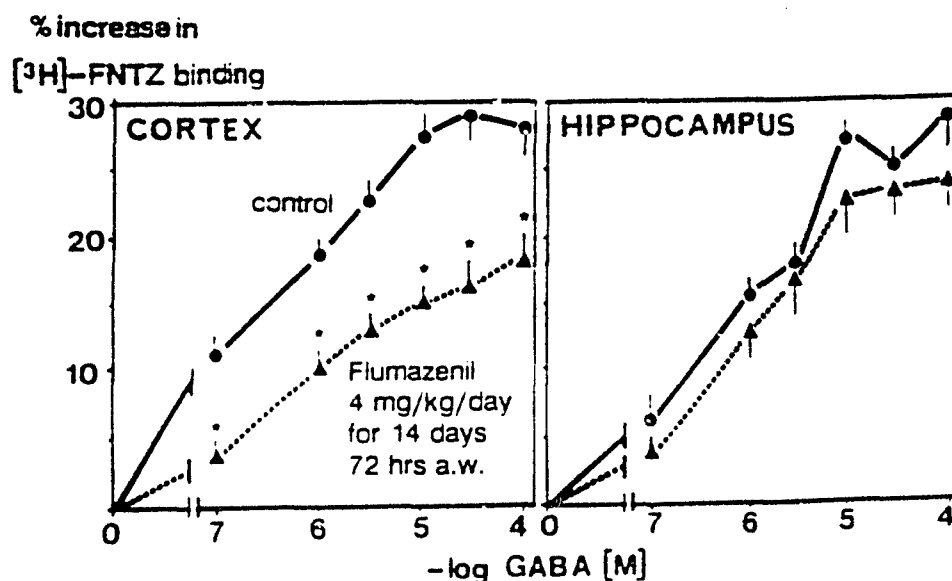


Fig. 2. Effect of GABA on [^3H]-flunitrazepam binding to cortical and hippocampal membranes from control (●) and rats chronically exposed to flumazenil (▲) (4 mg/kg per day for 14 days), 72 h after drug withdrawal. The binding assays were performed without or with GABA (0.1-100 μM) and in the presence of 0.6 nM [^3H]-flunitrazepam. Results are expressed as percent increase above basal values, i.e. in the absence of GABA. In the cortical membranes from flumazenil-treated rats, GABA facilitation of [^3H]-flunitrazepam binding was significantly reduced ($F(1,12) = 24.6$; $p < 0.0005$, ANOVA test), while in the hippocampal membranes there was no significant alteration in the GABA effect ($F(1,12) = 2.58$; $p = 0.13$). Values represent means \pm S.E. of seven animals/group (modified from Urbancic and Marczyński, 1989).

Disucssion

In the present study of rats chronically treated with FL, there were four major findings which are discussed below: 1) an increase in the non-appetively motivated exploratory behavior (NAMEB) which gradually emerged between day 5 and 7 of FL treatment and peaked at day 3 after FL withdrawal (its longer duration was not studied); 2) the NAMEB was correlated with decline in the defecation/urination scores; 3) a stabilization of the animals' goal-directed behavior, as measured by the "working memory" errors, when, after drug withdrawal, the animals were challenged by the introduction of guillotine gates and a 10 sec confinement to the center platform following each alley choice; and 4) contrary to the conventional view that the integrity of the GABA_A/BDZ/(chloride ionophore) receptor complex is essential for the animals' emotional stability, a significantly greater stability in the face of challenging environmental stimuli was instead associated with FL-induced uncoupling between the GABA_A and the BDZ recognition sites in cortical membranes, as measured by reduction in the GABA potentiation of flunitrazepam binding to cortex.

Is NAMEB a plausible interpretation? In the present study, the drug treated animals made significantly more visits to unbaited arms, i.e. responses that formally should be classified as "reference memory" errors (Olton and Pappas, 1979). However, we believe that it would be misleading to conclude that the animals had a deficit in the "reference memory", since their frequent visits to unbaited arms, facing the well illuminated "enriched" with furniture room environment were preferred over the baited alleys facing the room corner and suitable for hiding and/or escape. Thus, NAMEB is more likely to be related to the increased curiosity we have previously observed in FL treated rats in the Holeboard test, in which the holes with objects placed below strongly attracted attention of the FL-treated rats, but were largely ignored by the control animals (Urbancic et al. 1990). Interestingly enough, healthy human volunteers, following a single 5 mg i.v. dose of FL, reported a subjective feeling of a "pressure to move around" which was associated with EEG signs of increased vigilance (Schopf, Lurian and Gaillard, 1984).

One can plausibly argue that NAMEB reflects anxiolytic action of FL: 1) in rodents the defecation scores are indicative of emotional tension and temporary imbalance in the autonomic nervous system (Broadhurst, 1969; Sepinwall and Cook, 1982); and 2) in their home cages, the control and the drug treated rats consumed comparable amount of food and water (see the Methods). Thus, the low defecation scores in the drug group, and the inverse correlation of these scores with NAMEB may be interpreted as indexing greater emotional stability and the ability to habituate to novel environmental stimuli.

NAMEB and tonic changes in synaptic functions. The NAMEB emerged gradually between days 5 and 7 of chronic FL treatment and continued to increase, reaching its peak at day 3 after drug withdrawal, i.e. the time when we have found in the present and the previous study (Urbancic and Marczynski, 1989) that such a drug regimen upregulates the receptor density and affinity in the neocortex and hippocampus, and uncouples the link between the BDZ and the GABA_A recognition sites in the cortex (see Introduction; Urbancic and Marczynski, 1989). Similar FL-induced receptor and behavioral alterations have been reported in mice, in which chronic FL uncoupled the GABA/BDZ recognition sites, increased density of the BDZ receptors, and also upregulated the binding of the chloride channel ligand, t-butylbicyclopheosphorothionate (TBPS) at day 7 and 14, but not at day 1, 2 and 4 of chronic FL treatment (Miller et al. 1989).

Since the anxiolytic action of chronic FL outlasts for at least 10 days the 21 day drug treatment (Urbancic et al. 1990), it is plausible to expect that other yet unknown changes at the pre- and postsynaptic sites result from chronic FL treatment. For instance, chronic FL could reduce the availability of the endogenous angiogenic peptides derived from the Diazepam Binding Inhibitor (DBI) which are colocalized in and co-released with GABA (Ferrarese et al., 1987). In theory, chronic FL treatment can be expected to reduce the DBI synthesis and utilization, as such a process would be a logical counterpart to the effect of protracted administration of diazepam which was shown to increase the DBI synthesis and utilization in the cerebellum and cortex (Miyata, Mochetti, Ferrarese, Guidotti and Costa, 1987), an effect which could contribute to the development of tolerance to diazepam and withdrawal symptoms. This conjecture is supported by the observation that a single dose of FL has a peculiar capacity of reversing, for several days, the tolerance to anticonvulsant effects of chronic diazepam (Gonsalves and Gallagher, 1988), as if displacing a DBI-derived octadecano peptide (Ferrarese et al. 1987).

Potential metabolic effects of FL. Chronic FL strongly augments the time adult rats spend in REM sleep, without significantly affecting the slow wave sleep patterns; this action has a 4-6 day latency and outlasts for several days the time period of drug administration (O'Connor, Urbancic, Radulovacki and Marczyński, unpublished). The emergence of REM sleep is known to depend on a phasic surge in the tone of the ascending cholinergic and monoaminergic system and is associated with increased energy utilization and protein synthesis in the brain (Marczyński, 1989). Thus, chronic FL is likely to have disinhibitory influences not only on the cholinergic but also on the ascending brainstem monoaminergic systems which, like the forebrain cholinergic neurons (Zaborsky, 1986; Ulfig, 1988) receive a physiologically tonic GABAergic inhibitory input (Marczyński, 1986) utilizing the postsynaptic GABA_A/BDZ receptors (Osmanovic and Shefner, 1990; Gonsalves and Gallagher, 1988).

One should bear in mind that the brainstem ascending aminergic projections control the normal glia/neuronal metabolic relationships and energy utilization (Hertz, 1989). Thus, it is not surprising that FL increases the rate of cerebral glucose utilization (Richards, Burghard, Lorenz and Mohler, 1988), probably by attenuating the tonic physiological inhibition of the brainstem aminergic and cholinergic neurons projecting to the forebrain. Such an action, can be expected to improve chronically impaired metabolism in epileptogenic foci, and would account for FL's anticonvulsant action in humans, an effect which is unique in that it shows no tolerance and is not associated with soporific nor sedative effects (Scollo-Lavizzari, 1988).

Uniqueness of flumazenil's pharmacological profile. The clinically effective anxiolytic BDZ drugs are known to decrease vigilance, cognitive process, memory (Weingartner, 1985) as well as acquisition and retention of habituation to novel environmental stimuli (File, 1976; Heise, 1984). The BDZ anxiolytics share these actions with CNS depressants and anticholinergic drugs, such as scopolamine and atropine (Carlton and Vogel, 1967). On the other hand, chronic administration of FL has anxiolytic actions, while improving vigilance and cognitive processes (Urbancic et al., 1990), particularly, if the task (a two-stage swim-escape) requires a rapid analysis of environment (Urbancic, Gadek and Marczyński, 1991). FL also protects against amnesic action of scopolamine (Lal, Kumar and Foster, 1988; Urbancic et al. unpublished) and facilitates habituation to novel environmental stimuli if they are presented during the rat social interaction test (Urbancic et al. unpublished).

The cognitive and habituation processes are functionally interdependent

(Konorski, 1967; Kandel, 1982) and both are contingent on central cholinergic transmission and muscarinic receptors (Carlton, 1968; Karczmar, 1977; Marczynski, 1986; Mesulam, 1988). Hence, the aforementioned FL facilitation of cognitive processes and habituation is likely to be brought about by the FL-induced elevation in the tone of the cholinergic system. In support of this conjecture, a drug regimen similar to that in the present study facilitated, by a factor of 3, the acquisition and retention of the two-stage swim escape task in rats, while in the retention tests, FL antagonized the amnesic action of scopolamine (Urbancic et al. unpublished). Also, in mice a single but 5 times larger FL dose (20 mg/kg, i.p.) than that used by us chronically enhanced acquisition of avoidance behavior and protected the animals from amnesic action of scopolamine (Lal et al. 1988).

FL versus β -carboline BDZ receptor ligands. Although the cognitive and vigilance enhancing actions of FL appear to be comparable to those reported for some of the inverse BDZ agonists of the β -carboline group (Sarter and Stephens, 1988), contrary to FL, these drugs upon single or repeated administration, do not facilitate habituation to novel sensory stimuli (File and Pellow, 1988); instead they actually sensitize the animals to novel both neutral and anxiogenic stimuli, and may have proconvulsive actions (Grecksch, de Carvalho, Venault, Chapouthier and Rossier, 1983; Corda, Giorgi, Mele and Biggio, 1987). The interesting aspect of these adverse effects is that they may outlast the drug treatment by up to 20 days (Corda and Biggio, 1986; Corda et al. 1987), and therefore are qualitatively comparable to the effect caused by a strongly stressful experience (Antelman et al. 1988).

In conclusion, when compared to the β -carboline ligands, chronic FL seems to have a much greater clinical potential, as it uniquely combines anxiolytic, vigilance, cognitive and habituation enhancing properties, all of which result in an improved adaptive behavior.

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PART IV

"Chronic flumazenil (Ro 15-1788) facilitates acquisition and retention of a swim-escape response in rats". Urbancic, M., Gadek, M.A. and Marczynski, T.J., *Neuropsychobiology*, 1991 in press

Abstract

Since chronic flumazenil treatment was previously found to stimulate exploratory behavior in rodents, the aim of this study was to test the effect of chronic exposure to flumazenil on acquisition and retention of escape behavior. Adult rats were treated with flumazenil (Ro 15-1788; 4 mg/kg/day in drinking water) for 21 days (experiment 1) and for 17 days (experiment 2). In experiment 1 (a round water tank with one escape rope) conducted 24 hr after drug/vehicle withdrawal, the time the animals needed to resolve a swim escape task was significantly shorter in the drug group, compared to the controls. In the retention trial, 24 hr later, the control group matched the performance of the drug group. In experiment 2, a water T-maze was used which was equipped with two ropes, one anchored to the bottom and the other unanchored and therefore was more difficult to climb. On day 14 of flumazenil/vehicle treatment, there were no differences between the groups in time needed to escape from the maze. However, on day 15 and 16 of drug/vehicle treatment, the drug group made highly significant progress, while the control group showed no improvement of the escape behavior. The possible mechanisms of flumazenil-induced facilitation of escape behavior have been discussed.

Introduction

The imidazodiazepine Ro 15-1788 (flumazenil), a benzodiazepine (BDZ) receptor antagonist, was shown to be useful in reversing unconscious [1] and conscious sedation [2] caused by clinically proven BDZ anxiolytic drugs. Also, acutely administered flumazenil was found to improve hepatic encephalopathy [3], probably by blocking receptors for endogenous BDZ-like substance(s) present in the cerebrospinal fluid of patients with hepatic encephalopathy [4]. In mice, pretraining injection of flumazenil (2.5-40 mg/kg) was found to facilitate acquisition and retention of discriminated escape responses, and a single larger dose (40 mg/kg) of flumazenil was shown to provide protection against scopolamine-induced amnesia in a passive avoidance learning task [5]. These results led to the suggestion that flumazenil enhances memory by reducing the normally present GABA/BDZ receptor mediated inhibitory modulation of the basal forebrain cholinergic neurons [5] which are known to project to the neocortex and the limbic system and play a critical role in cognitive processes [6, 7]. Hence, flumazenil should be of interest in the potential treatment of cognitive impairments characteristic of Alzheimer's disease, a disorder known to primarily involve a dysfunction and degeneration of the forebrain cholinergic system [7].

We believe that chronic rather than acute flumazenil treatment may be relevant for achieving a sustained and well defined clinical effects; this is indicated by several recent studies: 1) chronic administration of flumazenil to pregnant and subsequently lactating dams, generated an adult rat progeny with

enriched hippocampal BDZ receptors, and this apparently enduring change was associated with more efficient goal-directed behavior [8, 9]; 2) in adult rodents, chronic exposure to flumazenil induced upregulation of BDZ receptors [10, 11, 12] and chloride channel binding sites [11], and reduced the ability of GABA to stimulate [³H]flunitrazepam binding to neocortical membranes [12]; 3) behavioral consequences of chronic flumazenil treatment of adult rats are different from acute administration, since during and following a 21 day treatment, no signs of anxiogenic action were noted; instead, during the third week of treatment we have observed increasing exploratory activity in the elevated plus-maze, indicative of anxiolytic action, and an anticonflict effect in the Vogel's punished drinking test for 10 day after flumazenil withdrawal [13]. An increased exploratory activity of rats in an elevated plus-maze [13] and in the radial-arm maze (Urbancic et al., unpublished data), as well as enhanced open-field activity in mice [11] were found to coincide in time with the BDZ receptor upregulation during chronic flumazenil treatment.

In the present study, our aim was to answer three questions: 1) is the anxiolytic effect of chronic flumazenil associated with impairment of memory and learning that is characteristic of BDZ receptor agonists [14]?; 2) is the aforementioned enhancing effect on learning after single exposure to flumazenil [5] also present during chronic treatment? and 3) is this effect still present after drug withdrawal, i.e. when the densities of BDZ receptors in cortex and hippocampus are increased and there is an uncoupling between the GABA and BDZ receptors in the cortex [12]? The latter question is of theoretical significance since, owing to the very short (16 min.) half-life time of flumazenil in the rat brain [33], this is the time when the increased vigilance and exploratory behavior remain unabated for up to 10 days after drug withdrawal [13], and, in the absence of the drug, the altered GABA/BDZ/chloride ionophore receptor complex is presumably no longer occupied by the drug and therefore "free" to interact with endogenous ligands [18, 19, 21]. Thus, in an attempt to answer these questions, in the third week of flumazenil treatment and following drug withdrawal, adult rats were tested for the acquisition and retention of escape behavior from a water tank and from a water T-maze.

Methods

Forty-two male Sprague-Dawley rats (Sasco-King, Omaha, NE), 70 days old and weighing 300-330 g at the beginning of the study, were used; they were singly housed in an air-conditioned room with a 14 hr light/ 10 hr dark cycle. After the average volume of the animals' water intake per 24 hr was ascertained, flumazenil was dissolved in ethylene glycol (5 ml per 1000 ml of tap water) and administered in calibrated drinking bottles for 21 day in the Experiment 1 and for 17 days in the Experiment 2. The volume of consumed water and body weight were measured every 24 hr and any significant change in water consumption was compensated by adjusting the drug concentration. The average daily dose of flumazenil equaled 4.0 mg/kg \pm 0.2 SD. The control group received a comparable volume of the drug vehicle in drinking water.

Experiments were conducted between 0900 and 1200 a.m. and all trials were videotaped for future replay and analysis. Two separate control groups and two drug groups of rats were selected on the basis of comparable weight.

Experiment 1. Active avoidance response was quantified using the swim escape test 24 and 48 hr after termination of a three week flumazenil treatment

(n=10 rats for each group). A circular plastic tank, 40 cm in diameter and 60 cm high, filled with water (25°C) to a depth of 25 cm was used. A thick rope (1 cm in diameter, 60 cm long) was suspended from an overhead steel bar (85 x 2 x 2 cm) into a center of the tank and held at the bottom with a heavy weight. A single acquisition trial was conducted 24 hr after drug withdrawal. The rat was slowly immersed into water, facing a fixed point at the tank circumference, and was allowed to swim until he climbed to the overhead bar or until 5 min have elapsed, whichever came first. The escape from the water tank was divided into two stages, and two time intervals were measured from the onset of each trial: first, the time required to climb the lower end of rope, holding the body out of the water (escape latency A), and second, the time to climb the additional 30 cm of rope to the overhead bar as a final task resolution (escape latency B). The retention trial was performed 24 hr following the acquisition trial. After termination of each trial, the animal was dried with a towel and placed into a cage under the heating lamp, before being returned to its home cage. The rationale for dividing the escape response into two phases, Goal A (grasping the rope) and Goal B (climbing to the overhead bar) requires two different levels of motivation, motor skills and/or astuteness in appraising the immediate environment.

Experiment 2. In another two groups of rats (n=11 for each group), the acquisition and retention of a swim escape response in the water T-maze were tested on days 14 through 16 of continuous flumazenil/vehicle treatment (trials 1 through 3), and on days 3 through 5 following drug withdrawal (trials 4 through 6). The testing apparatus was a water T-maze, with the stem (20 cm wide and 55 cm long) made of steel and two arms (29 cm wide and 32 cm long) made of fiberglass. The T-maze was 45 cm high and filled with water (25°C) to a depth of 25 cm.

In order to make the escape task more challenging, two ropes, 40 cm apart, were suspended from an overhead bar (85 x 2 x 2 cm, 35 cm above water level) into the center of each arm. One rope anchored to the bottom with a heavy weight, while the other rope was unanchored and therefore more difficult to grasp and climb. Powdered milk was dissolved in water to prevent visual discrimination between the anchored and the unanchored rope. At the onset of trial, the animal was placed in the stem of the T-maze and challenged to escape from the arm to an overhead bar (goal B) via a rope. An intermediate goal A was achieved when the animal grasped the rope and climbed to the lower end of rope, having all four legs above water level. In trial 5, the location of the anchored and the unanchored ropes had been reversed, in order to test relearning after drug withdrawal. Since the animals' ability to climb the unanchored rope was improving through the trials, in trial 6, the unanchored rope was made totally unclimbable, by putting it into a glass pipet.

The following measures were used to assess the performance of the animals: the time to reach goal A and goal B (the maximum allotted time was 10 min), and the numbers of re-entries into the stem of the maze from which there was no escape ("reference memory" errors). Like in experiment 1, the rationale for dividing the animal's performance in reaching goal A and goal B was that the latter clearly required a higher level of motivation and/or foresight in evaluating the environment, thus making the test more sensitive.

Statistics. In view of the exponential distribution of active avoidance data, we used the Cox/Mantel survival analysis test which was shown to be more appropriate than tests of significance that relied on measures of central

tendency, the median and the mean [15]. The analyses were carried out using the microcomputer software (Dr. J. Hintze's NCSS package, version 5.3, Kaysville, Utah, USA).

Results

Experiment 1 (round water tank). In the acquisition trial (24 hr after drug withdrawal; Fig. 1 top), there was no difference between the groups in the escape latency A (control: 75 ± 16.4 SE sec; filled circles, continuous line; flumazenil: 83 ± 17.1 SE sec; filled squares, dashed line). However, once on the lower end of rope with a body out of the water, the animals that had been treated with flumazenil, relative to controls, displayed a significantly increased motivation and/or ability to escape along the rope to the overhead bar ($p < 0.03$, Cox/Mantel survival analysis; Fig. 1, top; open circles vs. open squares). Specifically, while the control rats needed about 125.4 ± 35.3 SE sec from the moment they climbed the lower end of rope to the moment they reached the overhead bar, the flumazenil-exposed rats needed only 34.5 ± 15.2 SE sec. Therefore, the escape latency A and latency B were not different in the drug group ($p = 0.3$), while in the control group the escape latency B was significantly longer than the escape latency A ($p < 0.004$).

Relative to the acquisition trial (Fig. 1 top), in the retention trial (48 hr after drug withdrawal; Fig. 1 bottom), both groups showed a significantly improved performance and much shorter escape latencies A and B (control: $p < 0.005$, flumazenil: $p < 0.002$). In the retention trial, there were no differences between the groups in the escape latency A ($p = 0.7$) and escape latency B ($p = 0.9$; Fig. 1, bottom), mainly because the controls reduced the difference between the escape latency A and latency B from the 125.4 ± 35.3 SE sec (in acquisition trial) to 33.1 ± 20.8 SE sec ($p < 0.05$), thus reaching the performance level that had already attained by the drug-exposed rats (34.5 ± 15.2 SE sec) 24 hr earlier in the acquisition trial (Fig. 1 top).

% of animals failing to reach goal

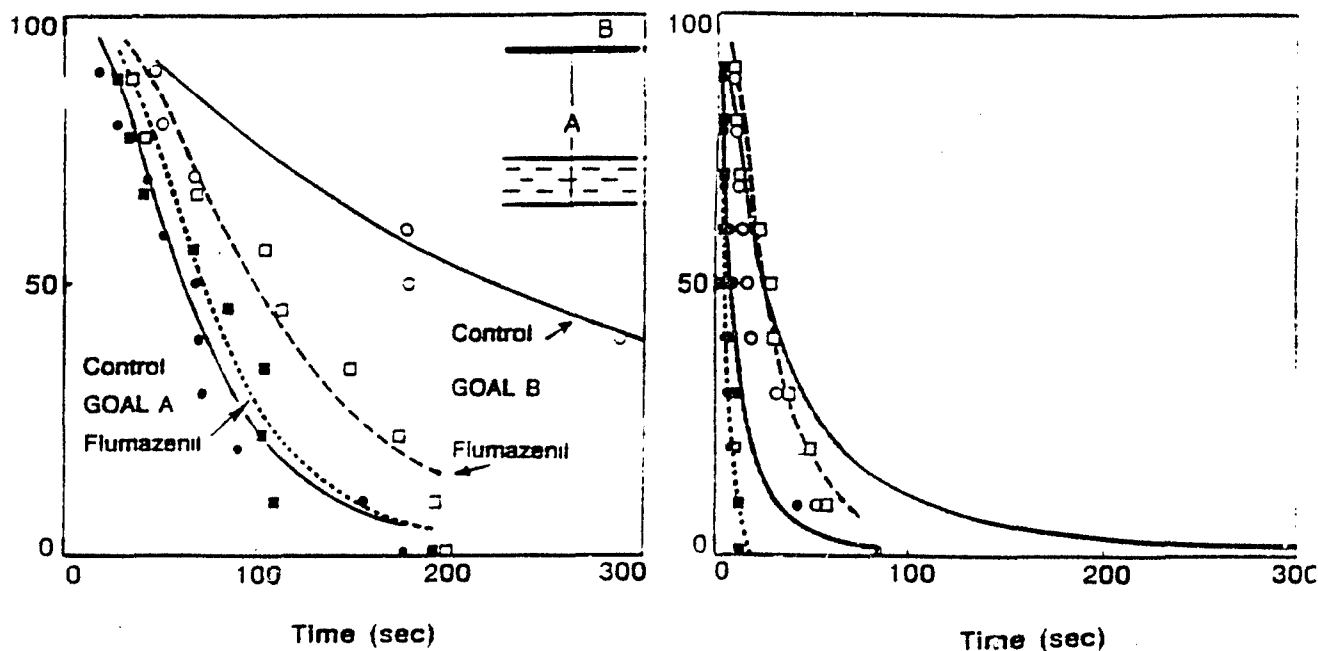


Fig. 1. Facilitation of resolution of water escape task (round water tank with one rope) following chronic, 21 day flumazenil (4 mg/kg/day) treatment, as tested 24 hr (acquisition trial, left) and 48 hr after flumazenil withdrawal (retention trial, right). The percent of animals that failed to reach goal A and goal B (ordinate) are plotted as a function of time (sec) from the onset of trial (abscissa). During the acquisition trial, the flumazenil-exposed rats ($n=10$; open squares-dashed line), compared to controls ($n=10$; open circles-continuous line), displayed enhanced ability to escape from the water tank to the overhead bar (goal B, $p<0.03$); there was no difference between the groups in reaching goal A (climbing the lower end of rope; control group: filled circles-continuous line; flumazenil group: filled squares-dashed line). Apparently, the "decision" to reach goal B was virtually instantaneous in the flumazenil group, since there was no difference between mean latency to reach goal A and goal B ($p=0.3$), while in the control group, this difference was highly significant ($p<0.004$). There were no group differences in motor skills per se, since, in the retention trial, both animal groups, relative to the acquisition trials, showed comparable performance improvements, markedly reducing the time necessary to reach goal A and goal B. For statistical data, see text.

Experiment 2 (water T-maze; Fig. 2). As shown in Table 1, in trial 1 (day 14 of flumazenil/vehicle treatment) there was no difference between the two groups in reaching goal A nor goal B ($p=0.53$ and $p=0.21$, respectively; the Cox-Mantel survival analysis). When comparing three trials (Table 2), relative to trial 1, in trial 2 and 3 (day 15 and 16 of treatment) the drug group significantly improved its performance in reaching goal A ($p<0.002$ and $p<0.00001$, respectively); this was also true for goal B ($p<0.04$ and $p<0.00001$, respectively) (Fig. 2, bottom). In contrast, the controls did not, or did marginally, improve their performance in trial 2 and 3 (Table 2 and Fig. 2, top). As shown in Table 1, in trial 3, there was no difference between the groups in reaching goal A ($p=0.26$), but the drug group, compared to control, continued to improve and significantly reduced the latency to reach goal B ($p<0.009$).

In trials 1 through 3, the flumazenil-treated group, relative to control, made fewer "reference memory" errors, i.e. the number of re-entries into the stem of the T-maze [$F(1, 120)=5.8$, $p<0.02$] from which there was no escape. In trial 4 through 6 (day 3 through 5 after drug withdrawal), there was no difference between the groups in latency to reach goal A nor goal B. However, in trial 6, relative to trial 4, the replacement of the unanchored but climbable rope with a glass covered and therefore unclimbable rope confounded the control group and increased their latency to reach goal A ($p<0.03$), while the flumazenil-exposed rats were not affected by this change ($p=0.13$) and rapidly recognized the change and focused their efforts almost exclusively on the opposite and climbable rope.

The numbers of "reference memory" errors, i.e. visits to the stem of T-maze, were not different between the groups in trial 4 through 6. However, in trial 6, relative to trial 4, the control group was clearly disturbed by the introduction of the unclimbable glass-covered rope and made significantly more "reference memory" errors [$F(1,120)=4.28$, $p<0.05$], while the performance of the flumazenil group remained unaffected by this novel adverse condition [$F(1,120)=0.0$, $p=1.0$].

Table 1

Statistical data for for Fig. 2 (water T-maze experiment). Effect of chronic flumazenil or drug vehicle treatment on between group differences in resolving the escape task from the water T-maze. Comparisons between the two groups are based on percent of animals that failed to reach Goal A and Goal B as a function of time (the Cox-Mantel survival analysis).

Flumazenil group vs. control group		
Trial #	GOAL A	GOAL B
1	$p=0.53$	$p=0.21$
2	$p=0.81$	$p=0.40$
3	$p=0.26$	$p<0.009$

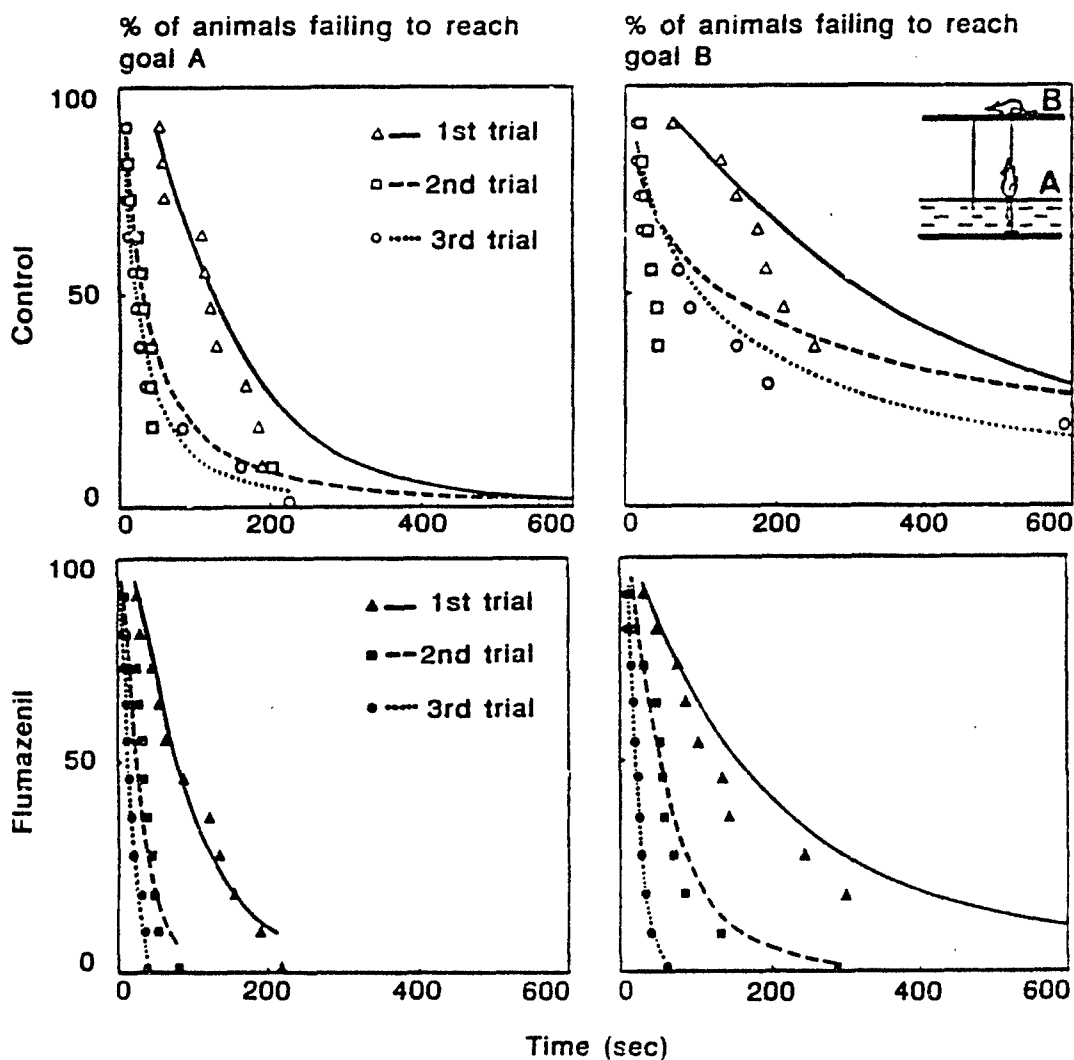


Fig. 2. Facilitation of resolution and retention of the water T-maze escape task by rats chronically treated with flumazenil (4 mg/kg/day). The percent of animals that failed to reach goal A and goal B (ordinate) are plotted as a function of time (seconds; abscissa) from the onset of trial. In trial 2 and 3 (day 15 and 16 of drug/vehicle treatment), the drug group (n=11; bottom plots) significantly improved their performance in reaching goal A and goal B, relative to trial 1, while the control rats (n=11; top plots) did not, or did marginally, improve their performance (for statistics, see the text and Table 1 and Table 2).

Table 2

Additional statistical data for Fig. 2; water T-maze tests. Effect of chronic flumazenil or drug vehicle treatment on within-group progress in acquiring the water escape behavior, i.e. reaching the intermediate Goal A and the final Goal B, over sequential three daily trials; the p values are based on the Cox-Mantel "survival analysis", i.e. percent of animals that failed to reach Goal A and Goal B as a function of time. For each group n=11.

	GOAL A			GOAL B		
	Compared trial numbers			Compared trial numbers		
	2 vs 1	3 vs 2	3 vs 1	2 vs 1	3 vs 2	3 vs 1
Control	p=0.07	p=0.51	p<0.05	p=0.36	p=0.48	p=0.18
Flumazenil	p<0.002	p<0.03	p<0.00001	p<0.04	p<0.004	p<0.00001

Discussion

Experiment 1 (round water tank). In the swim-escape acquisition trial carried out 24 hr after drug/vehicle withdrawal, the rats that had been chronically treated with flumazenil displayed a superior goal-directed behavior, compared to controls. There were no differences among the groups in the motor skills per se, since in the retention trial, 24 hr later, both animal groups showed comparable motor performance and significantly reduced the time necessary to reach goal A and goal B. The increased motivation and/or ability to climb the rope, and to reach the more distant goal B could have resulted from an anxiolytic effect of chronic flumazenil treatment [13] and/or from improved cognitive functions. This effect of flumazenil is analogous to the flumazenil induced unusually strong exploratory behavior of rats in the radial maze (Urbancic et al. unpublished); in this test, the drug group, when challenged by novel and "intimidating" environmental stimuli (introduction of alley guillotine gates to confine the animal for 10 seconds to the center platform), showed a much more efficient "working memory", despite that during the confinement period, the animals, compared to controls, were much more active, exploring all closed gates trying to lift them or climb over. Like in the present experiments, the most interesting aspect was that the flumazenil's effects, which emerged during the second week of treatment, reached its peak at the end of the 3rd week and remained unabated for 72 hrs after drug withdrawal (longer effects were not investigated).

Experiment 2 (water T-maze). Data obtained from trials conducted during flumazenil treatment clearly indicated enhanced learning of the swim escape task. Furthermore, following drug withdrawal, when both groups acquired similar level of performance, a change in the test apparatus (introduction of a glass-covered unanchored rope on day 5 of drug withdrawal) significantly disturbed the control rats, but failed to affect the drug group. Since the animals were tested daily and had become familiar with the test procedure, it is not likely that fear interfered with performance of the control rats. A more plausible explanation of superior performance

of the drug group is that it resulted from improved cognitive functions.

General discussion. We may conclude that the previously reported by us anxiolytic effect of chronic flumazenil treatment [13] is not associated with memory impairment, which is an untoward action linked to both acute and chronic BDZ-induced anxiolysis [14, 16, 17]. On the contrary, chronic flumazenil seems to combine anxiolytic action with improvement of acquisition and retention of novel aversively motivated goal-directed behavior. This nootropic action may be linked to the flumazenil enhanced brain metabolism, as revealed by oxygen utilization in the rat forebrain [32]. The nootropic effect may also be linked to increased protein synthesis in the CNS, an action which may be deduced from our recent observations that chronic flumazenil strongly increases the time rats spend in the Rapid Eye Movement (REM) sleep, an effect which was still present for at least several days after drug withdrawal (O'Connor et al. unpublished).

Based on our present data and results from other laboratories [5, 18], it appears that flumazenil-induced facilitation of learning can best be revealed in aversively motivated behaviors. These findings are in agreement with the suggestion that an endogenous ligand for BDZ receptors is released during stress and interferes with learning [18]. In line with this hypothesis are the findings that stress, such as forced swimming [19] or exposure to an elevated plus-maze [20], decrease BDZ receptor binding in the brain. An acute swim stress in rats was found to increase the cerebral cortex levels of an endogenous BDZ receptor binding inhibitor, which was identified as n-butyl- β -carboline-3-carboxylate [21]; this agent may have anxiogenic properties comparable to those of other β -carboline [22].

A moderate arousal or emotional tension may be necessary for learning [23], but an excessive orienting reaction and arousal may impair selective attention and learning. Consistent with this view is the finding that low and high doses of BDZ receptor inverse agonists of the β -carboline type, respectively, improve or impair acquisition of a passive avoidance task [24]. Hence, flumazenil could partially block the access to BDZ receptors of an endogenous β -carboline ligand responsible for hyperarousal and its interference with learning. However, the validity of this interpretation may be questioned on the basis of our previous [13] and present observations that the anxiolytic and nootropic actions of chronic flumazenil remains unabated for at least several days after drug withdrawal, when in the drug is presumably no longer present, owing to its rapid metabolism to a totally inactive compound [32] and a very short (16 minute) half-life time in the rat brain [33].

On the other hand, the ability of flumazenil to prevent the scopolamine-induced amnesia [5], indicates that flumazenil could enhance cognitive functions and memory by antagonizing the BDZ/GABA receptor mediated and normally present tonic inhibition of the basal forebrain cholinergic neurons [5, 25, 26, 40] whose functions are critical for cognitive processes in the cortex and the limbic system [6, 7].

The validity of our tests for nootropic action of flumazenil is enhanced by the fact that in other water maze designs [30], 25 months old rats, compared to 3 months old animals, make significantly more errors and need much more time to learn and remember the location of the exit ladder, the effect of age being more apparent with increasing complexity of the maze [31].

In conclusion, the present and previous observations [13] indicate that chronic flumazenil has a unique pharmacological profile, since it seems to have anxiolytic actions, while enhancing vigilance and cognitive processes. We suggest that these actions may be, in part, due to the elevation of brain metabolism and protein synthesis, as indicated by our observations that rats chronically treated with

flumazenil spend much more time in REM sleep than the control animals, an effect which gradually wanes over several days after drug withdrawal (O'Connor et al. unpublished). Hence, it appears that flumazenil, similarly to the antagonist β -carboline ZK 93426 [26], has a much greater clinical potential for the treatment of chronic cognitive impairments than, for instance, the β -carboline inverse BDZ agonists [27], for the latter, if chronically administered, are known to sensitize the CNS to their proconvulsive action [28] and to anxiogenic environmental stimuli [29], while flumazenil, in addition to its vigilance enhancing and nootropic actions, has antiepileptic properties in humans [34].

Not only the forebrain cholinergic system [40], but also the ascending brainstem cholinergic [36] and the aminergic systems [35, 36, 37] are controlled by tonic inhibitory GABAergic neurons and GABA/BDZ receptors. These systems are indispensable for normal glia/neuronal metabolic relationships [38] and REM sleep [39]. Hence, in order account for all of the aforementioned "energizing" and nootropic properties of chronic flumazenil, including its anticonvulsive actions [34], we propose that these actions result from disinhibition of the ascending aminergic and cholinergic systems, and that the disinhibition of cholinergic neurons may take place both at the brain stem nuclei and the basal forebrain and septal neurons. The gradually emerging disinhibitions of these systems during chronic administration of flumazenil may be induced by partial uncoupling between the GABA_A and the BDZ recognition sites, for we believe that the uncoupling found by us in rat cortex [12] may also develop in the basal forebrain and the brain stem.

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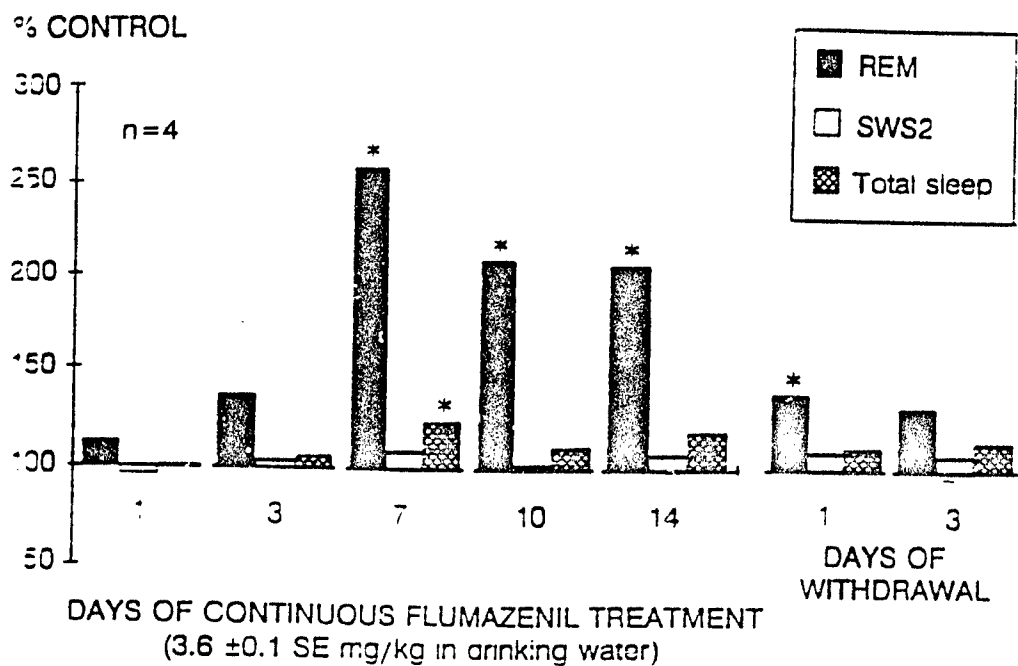
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PART V

Chronic RO 15-1788 (Flumazenil) treatment increases REM sleep in rats:
Sean O'Connor, M. Urbancic, T.J. Marczyński and M. Radulovacki, in preparation

Abstract

Chronic administration of Ro 15-1788 (flumazenil; average dose 3.6 mg/kg/day in drinking water) elicited a gradual increase in the total time the animals spent in REM sleep. Enhanced REM sleep was still observed for 3 days after drug withdrawal. There were no significant changes in the slow wave sleep patterns (Fig. 1 below).



PART VI .

Benzodiazepines inhibit neutrophil chemotaxis and superoxide production in a stimulus dependent manner; PK-11195 antagonizes these effects: Molly Finnerty, T.J. Marczynski, H. Amirault, M. Urbancic, B. Andersen, Immunopharmacology, submitted,

Abstract

Diazepam, which binds both central (neuronal) and peripheral (non-neuronal) benzodiazepine receptors, and Ro5-4864, a ligand selective for peripheral benzodiazepine receptors, both inhibited the FMLP induced chemotaxis in human neutrophils at concentrations as low as 10^{-8} M in some individuals. A selective peripheral benzodiazepine receptor antagonist, PK-11195 (10^{-5} M), partially reversed the benzodiazepine inhibition. Diazepam also inhibited the superoxide production induced by FMLP, NaF, and A23187, but not that induced by PMA; the FMLP-induced superoxide production was most sensitive to diazepam inhibition (ID_{50} , 2.25×10^{-6} M diazepam); the effect of NaF was slightly less sensitive (ID_{50} , 1.34×10^{-5} M diazepam); and the A23187-induced superoxide production was significantly inhibited only at 10^{-4} M diazepam concentrations. Like diazepam, Ro5-4864 inhibited the FMLP-induced superoxide production, and PK-11195 (10^{-5} M) partially antagonized both diazepam and Ro5-4864 inhibition.

Binding studies showed the presence of a peripheral benzodiazepine receptor on human neutrophils with a K_D of $1.2 \pm 0.06 \times 10^{-8}$ M (\pm SEM), and a B_{max} of 1028 ± 86.2 fmoles/ 10^6 cells (\pm SEM) for [3 H]Ro5-4864.